

Immunocytochemical studies in colorectal carcinoma

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IMMUNOCYTOCHEMICAL STUDIES IN COLORECTAL CARCINOMA

Jan Willem Arends

Immunocytochemical Studies in Colorectal Carcinoma

Proefschrift ter verkrijging van de graad van Doctor in de Geneeskunde
aan de Rijksuniversiteit Limburg
te Maastricht op gezag van de Rector Magnificus
Prof.Dr. H.C. Hemker volgens het besluit
van het College van Dekanen in het openbaar te verdedigen
in de Aula van de Universiteit op

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door

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geboren te Groningen.

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Prof. P. Isaacson, M.R.C. Path.

To the memory of my father

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Chapter I

INTRODUCTION.

In this chapter a review of the literature on the general and clinicopathological aspects, nature and methods for the detection of antigens in neoplastic tissue is presented. Subsequently, a classification of tumour antigens is proposed and the most relevant antigens with regard to colorectal carcinomas are described.

Both the possibilities of tissue antigen detection in respect of diagnosis, classification and assessment of prognosis in colorectal cancers and the limitations inherent to the approach in terms of antigen - specificity and phenotypic heterogeneity of antigen expression are discussed.

This chapter is concluded with a description of the design of the present study.

1.1. General value of tumour - antigen expression

Because of the perspectives inherent to the detection of compounds specific for or associated with neoplasia in body fluids and tissues the study of tumour-antigens has aroused great interest in many disciplines of medical science during the past decade.

The option for the clinical oncologist mainly lies in the measurement of tumour - antigens in body fluids for early diagnosis and the monitoring of the results of tumour therapy. In addition the perspectives for the ability to localize and possibly treat tumours through immunotargeting procedures are challenging.

Identification and histotopographic localization of substances, such as tumour - antigens, add additional criteria to those currently employed in histopathology for classification and assessment of histological grade in carcinomas.

Study of antigen expression at the cellular level is of value for the cell biologist to relate alterations in antigen display to the process of cellular differentiation and the mechanisms involved in the development of aberrant behaviour of neoplastic cells.

1.2. Clinicopathological value of tumour - antigen expression

For clinicopathological purposes the study of antigen expression in tissues is most relevant in regard of:

a. The characterization and classification of primary-tumours and metastases of unknown origin.

An excellent example of the value, which detection of antigens occurring in a limited number of tissues may have for tumour classification, is the immunohistochemical detection of prostate specific acid phosphatase. Due to the almost exclusive presence of this antigen in prostatic glandular epithelium and derivative neoplasms adenocarcinomas can be

characterized and metastatic lesions can be demonstrated as of prostatic origin (Jöbsis et al., 1978).

b. The assessment of the type and degree of differentiation of tumours (assessment of prognosis)

The value of determination of antigen display in morphologically identical neoplasms for defining subgroups with a distinct biological and clinical behaviour is exemplified in acute lymphatic leukemia (ALL). According to the expression of B and T cell antigens ALL has been separated into at least three groups: non B - non T cell ALL, T cell ALL and B cell ALL, each with a distinct clinical behaviour, response to therapeutic regimes and prognosis (Jondal et al., 1973; Tsukimoto et al., 1976; Belpomme et al., 1977; Brouet et al., 1978; Sallen et al., 1980; Greaves, 1981).

1.3. Nature of tumour - antigens

Since the early days of Ehrlich (1906) it has been postulated, that cells in the process of neoplastic transformation acquire tumour-specific antigens capable to elicit immune reactions, which might play a role in the defence of the organism against the development of neoplasia. The first conclusive evidence for the existence of such antigens in animals was obtained by Foley (1953), who demonstrated that methylcholantrene -induced tumours transplanted in syngeneic mice could be rejected. In the following years similar observations were made in various virally and chemically induced tumour systems in experimental animals (see for review Baldwin, 1973).

Evidence for the existence of tumour-specific antigens in humans, however, remained circumstantial for various reasons. Firstly, due to the ethical preclusion of in vivo studies, demonstration of cellular and/or humoral immune responses in cancer patients against their tumour only relied on elaborate in vitro studies, in which autologous tumour cells seldom could be used. It, therefore, remained impossible to definitely rule out the possibility, that the immune reactions observed were directed against alloantigens present in the tumour - test - tissue used in these experiments. Secondly, the identification of tumour-specific antigens by immunizing with human cancer cells or products of cancer cells has been frustrated by the strong immunogenic challenge by normal human tissue antigens present on the immunizing agents. This problem necessitated extensive absorption of xenoantisera with normal human tissues. During this process, however, trace amounts of tumour-specific antibodies could have been lost as well.

The hybridoma technique introduced by Kohler and Milstein (1975), which enables the generation of unlimited amounts of monospecific antibodies and also potentially allows for the selection of antibodies directed against weak immunogens, has created the expectation that the problem, whether or not antigens unique to a given tumour and not expressed in other tumours or normal tissues occur in the human situation, would be answered.

This expectation, however, as yet has not been substantiated and so far experimental data suggest, that in respect of antigen expression, neoplastic tissue only differs from normal tissue in a quantitative, but not in a qualitative sense. In view of this situation it might be more appropriate to speak of tumour - associated antigens than of tumour - specific antigens. Many of these antigens, such as carcinoembryonic antigen, have been found predominantly in neoplastic cells and, although in almost all cases extensive studies demonstrated their expression in non-neoplastic cells as well, we will refer to these antigens as "tumour-associated". Other antigens occur in normal cells, but have been studied in neoplastic cells, because they relate to particular lines of differentiation (such as the intermediate filament proteins) or are specific for a single cell type (such as thyroglobulin or prostate specific acid phosphatase) and will be referred to as "tissue-antigens". Although certain tumour cell products or constituents are detected by other than immunological methods, the designation tumour "antigens" in general appears to be acceptable in view of the fact that the majority of tumour-associated macromolecules are studied through their detection by specific antibodies.

Since this study concentrates on colorectal carcinomas, in the following paragraphs the discussion on tumour-antigens will be mainly restricted to antigens of relevance for tumours of the gastrointestinal tract and more especially the large intestine.

1.4. Methods for the detection of tumour-antigens in tissue

1.4.1. Conventional histochemical staining methods, which allow the visualization and localization of a category of substances rather than one chemically defined molecule have been employed by histopathologists since a long time. For tissues of gastrointestinal tract derivation histochemical reactions on mucosubstances are of special interest. Neutral mucins, sialomucins and sulphomucins can be visualized with the diastase-periodic acid-Schiff, the alcian-blue at pH 2.5 and high iron-diamine reactions respectively (Pearse, 1968; Spicer, 1965). A number of refined stains has been developed, which often permit distinction of different mucins in the same section (Filipe, 1979; Culling et al., 1976). Neuroendocrine cells in normal and neoplastic gastrointestinal mucosa can be detected with silver impregnation methods, such as the argentaffin and argyrophil staining reactions (Sevier and Munger, 1965; Grimelius, 1968).

1.4.2. Enzymehistochemistry, which is based on the interaction of a given enzyme with a given substrate allows specific identification of tissue enzymes (Willighagen, 1970). Due to the fact that most enzymes do not appear to be organ or cell specific, its practical use, however, is limited and this also applies to the gastrointestinal tract.

1.4.3. Immunocytochemistry nowadays is by far the most important method to identify cell products or constituents. The technique basically relies on the specific interaction between tissue antigens and corresponding antibodies, which can be visualized with various fluorochromes (Coons et al., 1941) or enzyme labels (Nakane et al., 1966; Avrameas, 1967). In the past 15 years numerous innovations and modifications of the original standard methods have been developed, which are summarized in many reviews (Taylor, 1978; Falini and Taylor, 1983; Bosman, 1983). Most of these alterations are aimed at improving the specificity and sensitivity of the technique.

Specificity problems may occur at the level of the antibody used and/or the method employed. Antibody-specificity is one of the most important conditions for reliable immunocytochemistry and should be ascertained by adequate control procedures. As a first step affinity purification of antisera with the purified antigen is mandatory. The specificity of the antiserum thus obtained should be further checked with immunodiffusion and immunoelectrophoresis or with more sensitive radio- or enzyme immunoassays. The hybridoma technique (Kohler and Milstein, 1975), which enables the generation of unlimited amounts of monoclonal and therefore monospecific antibodies, appears to be a major improvement in antibody-specificity, but the supreme specificity of these antibodies may in itself be a problem, due to the occurrence of their target-epitope on different non-chemically related macromolecules (Diamond et al., 1981; Dulbecco et al., 1981). Method specificity should be determined by adequate tissue controls involving the inclusion of positive reference tissues, omission and replacement of the specific antibody by non-immune serum and blocking with purified antigen (Dellelis et al., 1979).

The sensitivity of the technique has been enhanced by procedures to increase the number of enzym-labels per antigen binding site (Ordroneau et al., 1980) and also by intensifying the reaction product obtained with a given amount of enzymatic activity (Gallyas et al., 1982; Strauss, 1982). In respect of routinely processed formalin fixed and paraffin embedded tissues it should be realized that denaturation of antigenic structures due to tissue preparation procedures might compromise the sensitivity of the reaction. The extent of antigenic preservation appears to be variable for different antigens and largely depends on the fixative employed (Curran et al., 1980). Proteolytic digestion of tissue sections with e.g. trypsin prior to incubation with antibody may enhance sensitivity by "unmasking" antigenic sites and allow detection of many antigens after routine fixation with 4% neutral buffered formalin (Mephram et al., 1979).

1.4.4. Lectin-affinity reactions have been used to explore the occurrence of carbohydrates and glycoproteins on cell membranes in tissue sections (Stoward et al., 1980; Walker,

1982; West et al., 1982). Lectins bind glycoproteins through their affinity for specific sugar sequences. Many different lectins are known, each with specificity for a particular sugar sequence (for review see Gold and Balding, 1975). Staining of tissue with lectins can be performed either with direct lectin fluorochrome conjugates or using immunological procedures involving the application of a lectin antibody in a direct or indirect fluorescence test after prior incubation with the lectin (Leatham and Atkins, 1983). On paraffin sections lectin binding often appears to be attenuated due to denaturation of proteins with carbohydrate sequestration during the process of fixation and embedding. Prior trypsinization and/or neuraminidase treatment of the paraffin sections may considerably increase the intensity of the lectin binding reaction, but the pattern of distribution of the lectin binding carbohydrates may be altered after these procedures (Leatham and Atkins, 1983). Therefore lectin affinity reactions preferably should be performed in mildly fixed frozen tissue sections.

1.5. Classification of tumour-antigens

As indicated in paragraph 1.3. we propose a subdivision of tumour-antigens into tumour-associated antigens and tissue-antigens.

Although most of the tumour-associated antigens also occur in normal tissue components, the designation is chosen to indicate that antigens of this nature are predominantly found in neoplasms or are somehow related to the neoplastic process.

Tissue antigens, in contrast, comprise normal cell constituents or cell products, which may have relevance for the study of tumour tissue.

Of course it should be realized that the nature of certain antigens does not allow an unambiguous classification into one of these two main categories, so that the subdivision remains rather arbitrary. For example, some mucoproteins defined by conventional antibodies have been tested mainly in tumours and/or are considered to be of oncofetal nature. Nevertheless, they will be discussed in connection with the mucins detected by conventional histochemical reactions in the paragraph on goblet cell products.

In general, however, this subdivision seems to be rather logical and allows the classification, even of tumour antigens of ill-defined nature, in a satisfactory scheme in which relatively few overlaps, omissions or misplacements occur.

1.5.1. Tumour-associated antigens

Tumour-associated antigens may be distinguished into three sub-groups: nuclear, oncofetal and other antigens.

a. Tumour-associated nuclear antigens

In the area of research focussed on alteration of nuclear

(chromatin) antigens in neoplasms caused by environmental carcinogenic agents or oncogenic viruses a lot of experimental data has been collected by molecular biologists, which indicates the tremendous impact of these studies on our understanding of the genetic mechanisms involved in malignant transformation and also provides challenging perspectives for new approaches in the classification and treatment of neoplasia.

Tumour-associated nuclear antigens which seem to appear at an early stage of malignant transformation, have been demonstrated in 1-2-dimethylhydrazine-induced colonic tumours in rats (Chiu et al., 1980; Gabryelak, 1981) and the human colonic adenocarcinoma cell lines HT-29 and LoVo (Duhl et al., 1982). The existence of virally induced tumour-associated nuclear antigens has not been reported in respect of colonic carcinoma.

Recent studies using gene-transfer methodology (Weinberg, 1981) have shown the existence of genes activated in human tumours, which confer the properties of malignancy (Shik et al., 1979; Cooper, 1980). These genes, known as cellular transforming genes, or oncogenes, have been demonstrated in cell lines and cell preparations of surgically removed human cancers amongst which colorectal adenocarcinomas (Pulciani et al., 1982). The DNA sequence of most oncogenes now isolated from human solid tumours shows a remarkable homology to genes carried by one family of retroviruses (Pulciani et al., 1982; Santos et al., 1982; Parada, 1982; Shimizu et al., 1983). Other human transforming genes, particularly those from leukemias and lymphomas are known to occur due to chromosomal translocations (Rechavi et al., 1982; Taub et al., 1982; Dalla-Favera et al., 1982; De Klein et al., 1982) and have not been matched to any of the human viral oncogenes. Therefore experimental data so far suggest that activation of an oncogene can be due to mutation of a normal gene or chromosomal translocation of a gene, by the virtue of which this gene is more actively transcribed. Thus, the proteins encoded by transforming genes may consist of real tumour - neoantigens, but also of normal cell compounds, which are produced in quantities different from those in normal cells. Identification and manipulation of the transcription products of oncogenes may be expected to generate new impulses in all fields of cancer research, but the clinicopathological implications as yet seem to be remote.

b. Oncofetal antigens

Carcinoembryonic antigen (CEA), the prototype of colorectal carcinoma-associated antigens, originally was thought to be of oncofetal nature (Gold and Freeman, 1965; Gold and Freeman, 1965). Although its presence has now been detected in protein synthetic organelles and in a polar pattern at the microvilli of the apical plasmamembrane of normal columnar and goblet cells of colonic epithelium (Ahnen et al., 1982), the antigen will be discussed in this paragraph on

oncofetal antigens because of its strong historical association with antigens of this nature.

CEA has been most extensively studied and a number of reviews on the characteristics of the antigen are available, of which those of Rogers (1976 and 1983) present a complete and up to date description. In this paragraph information on CEA is restricted to its application at the tissue level and to some recent developments concerning the generation of CEA reactive monoclonal antibodies.

Due to its presence in normal colonic mucosa (Khoo et al., 1973; Goldenberg et al., 1975; Huitric et al., 1976; Isaacson and Judd, 1978; O'Brien et al., 1981), as well as carcinomas of e.g. respiratory (Pascal et al., 1977; Hill et al., 1979) and genitourinary tract origin (Marchand et al., 1975; Heald et al., 1979; van Nagell jr., 1979; Shousha and Lyssiotis, 1978; Shevchuck et al., 1981) the value of CEA in colorectal carcinoma has been reduced to its serum assessment for monitoring of colorectal cancer patients (Goldenberg et al., 1981; Persijn and Hart, 1981).

The lack of tumour specificity of CEA has been attributed to heterogeneity of the molecule as determined after various purification methods (Fuks et al., 1974; Vrba et al., 1975) and crossreaction of polyclonal antisera with other antigenically related molecules (von Kleist et al., 1972; Mach and Pusztaszeri, 1972; Burtin et al., 1973; Svenberg et al., 1979; Kuroki et al., 1982). Efforts to improve the specificity of CEA detection by more elaborate methods of purification or absorption of antisera with CEA-crossreacting agents have not yielded antisera with a more restricted reaction pattern. Recently, however, a rapid and reproducible immunoabsorption technique resulting in a monospecific anti CEA serum applicable in histopathology has been described (Nap et al., 1983).

Monoclonal antibodies should offer a tool to define the CEA molecule and associated molecular forms more precisely. Antibodies of this variety therefore should provide means to test the possibility whether different forms of CEA are expressed in neoplastic and normal tissue or in different conditions of disease (Rogers, 1976). In the past few years several groups of workers have reported on the generation of monoclonal antibodies reactive with CEA (Mitchell, 1980; Accolla et al., 1980; Kupchik et al., 1981; Rogers, 1981; Buchegger et al., 1982; Primus et al., 1983a), but few reports deal with their reaction pattern at the tissue level. Monoclonal antibodies have been claimed to be superior to conventional antisera in immunoperoxidase staining of CEA, due to their high affinity resulting in neglectable background staining (Lindgren et al., 1982). Primus et al., (1983b) described an anti CEA monoclonal antibody lacking nonspecific cross reacting antigen (NCA) reactivity, which reacted with only 30% of colorectal carcinomas and therefore may recognize a unique determinant expressed on a subpopulation of CEA molecules. Its staining of normal colonic mucosa was apparent, but weak. Imai et al. (1984) studied

three monoclonal antibodies reactive with CEA or CEA related molecules, which reacted with the majority of colon carcinomas and not with normal or fetal colonic mucosa. Wagener et al. (1983) reported on a monoclonal antibody, which demonstrated an almost exclusive specificity for a tumour-associated antigenic determinant on the CEA molecule and showed no or marginal crossreaction with biliary glycoprotein (BGP) and NCA respectively. Therefore, there are indications that CEA reactive monoclonal antibodies may probe into the problem of heterogeneity of the CEA molecule and associated crossreacting antigens more exactly, but before a clear picture about the precise state of purported specific CEA moieties in respect of different organs and conditions of disease is to be given, a lot more of documentary work is to be done.

In recent years much attention has been focussed on the development of techniques to detect and localize malignant neoplasms by means of radiolabeled antibodies in combination with scintigraphic scanning methods. Due to its uniform expression in the majority of tumour cells of both primary and metastatic colorectal tumours most immunotargeting procedures so far have used CEA as target-antigen despite its distribution in normal intestinal mucosa and its release into body fluids Goldenberg et al. (1980, 1982) have reported with optimism about the perspectives of immunotargeting procedures using anti CEA antibodies as targeting vehicles in transplant tumour bearing nude mice and colorectal cancer patients, whereas Mach and co-workers (Mach et al., 1981; Bercher et al., 1982) employing both conventional and monoclonal CEA reactive antibodies in the same experimental settings seem to be more cautious in their expectations. Others (Hedin et al., 1982) have also reported about the use of monoclonal anti CEA antibodies in immunotargeting.

The presence of alpha-fetoprotein (AFP) in about half of colonic carcinomas has been mentioned in one publication, but no details about its exact distribution were given (Skinner and Whitehead, 1981). Oncofetal antigen-1 (OFA-1) has been reported to occur in 15% of colorectal carcinomas (Rees et al., 1981). Other products associated with fetal tissues demonstrated in colorectal cancers include human chorionic gonadotropin (HCG) (Gailani, 1976; Buckley and Fox, 1979) and human placental lactogen (HPL) (Skinner and Whitehead, 1981). A fucosyl antigen expressed in colon adenocarcinoma and embryonal carcinoma cells has been recently reported (Miyauchi et al., 1982).

c. Other tumour-associated antigens

Some antigens, which have been studied in connection with colorectal cancer mainly or exclusively at the serological level are listed below and will not be discussed in greater detail, because of lack of experimental data concerning their expression in colorectal carcinoma tissue.

| <u>Designation</u> | <u>Abbreviation</u> | <u>Literature</u> |
|--------------------------|---------------------|---|
| Zinc glycinate marker | ZGM | Pusztaszeri et al., 1976 |
| Tennessee antigen | Tenna gen | Potter et al., 1976 Gray et al., 1982a, b; Pentycross et al., 1982 |
| B2 microglobuline | B2 M | Staab et al., 1980 |
| Galactosyltransferase II | GT II | Podolsky et al., 1978 |
| Membrane tissue antigen | MTA | von Kleist et al., 1974 |
| Basic fetoprotein | BFP | Ishii, 1979 |

Other antigens of potential interest in the study of colorectal carcinomas include substances of ill-defined nature associated with colorectal cancer cells mainly detected by monoclonal antibodies. The techniques involved in the generation of monoclonal antibodies nowadays almost have reached the standard routine level. As a result many reports have been published concerning monoclonal antibodies derived from immunization of mice with colonic carcinoma cell lines, freshly prepared colonic carcinoma cell suspensions or preparations of colonic cancer cells membranes, some of which are claimed to detect colorectal carcinoma-specific antigens. The main problem with monoclonal antibodies, however, lies in the often cumbersome characterization of their target-antigens by various biochemical procedures and the exploration of the distribution of these target-antigens by immunocytochemical methods on cell lines and tissue sections of various sources (Thompson et al., 1983).

The fate of the 1116 NS19-9 monoclonal antibody defined, gastrointestinal cancer associated antigen (GICA) nicely illustrates the pertinence of extensive biochemical and immunocytochemical characterization, before a monoclonal antibody can be hailed as detecting a tumour-specific antigen. Originally the target antigen was claimed to be of oncofetal and glycolipid (monosialoganglioside) nature (Magnani et al., 1981) and reported to be restricted to cancers of the colon, stomach and pancreas (Koprowski et al., 1979; Chang et al., 1981; Koprowski et al., 1981). Subsequently by immunoperoxidase studies its presence was detected in various normal adult glandular tissues as well as carcinomas of other than gastrointestinal tract origin (Atkinson et al., 1982). Part of this work has been done by our group and will be discussed in Chapter II (Arends et al., 1983). Recently the glycolipid nature of GICA has also become contested (Raux et al., 1983; Magnani et al., 1984). Monoclonal antibodies which have so far been reported in connection with colorectal cancer are compiled in table I, together with their characteristics. From this table it can

Monoclonal antibodies associated with colorectal carcinoma

| Designation | Immunizing agent | Specificity for colorectal cancer | Immunocytochemical characterization | Nature of antigen | Literature | |
|-------------|--|-----------------------------------|--|-------------------|--|---------------------|
| | | | cell lines | tissue sections | | |
| 1116 NS | | | | | | |
| 19-9 | SW colonic carcinoma cell line cells | | + | + | monosialoganglioside CEA 180.000 | |
| 52 a | | | + | | | |
| 3 d | | | + | | | |
| 10 | | | + | | LeB blood group | |
| 38 a | | | + | | | |
| 43 | | | + | | | |
| 3 a | | | + | | Neutral glyco-lipids | |
| 33 a | | | + | | | |
| 33 b | | | + | | | |
| WGHS | | | | | | |
| 9-1 | Freshly isolated gastric carcinoma cells | | + | | protein with chain KD 28.000 chain KD 22.000 lacto-N-fucose pentose III | |
| 9-2 | | | + | | | |
| 29-1 | | | + | | | |
| WCDK | | | | | | |
| series | SW colonic carcinoma cell line cells | | + | + | Steplewski & Koprowski (1982) | |
| YPC series | | | | | | |
| 2/12.1 | | | retained reactivity in paraffin sections | | | Finan et al. (1982) |
| 2/443. | | | + | | | |

| | | | | |
|------------|--|---|---|----------------------------|
| 1083-17 | SW colonic carcinoma | S | + | Herlyn et al. (1983) |
| 1116-56 | cell line cells | S | + | |
| 250-series | fresh colonic carcinoma | | | |
| 250.30.6 | cells + Ht-29 cells | | + | Thompson et al. (1983) |
| C-series | | | | |
| C50 | Colo-205 colonic | S | + | Lindholm et al. (1983) |
| C51 | carcinoma cell line | S | + | |
| C53 | cells | S | + | |
| B 72.3 | Membrane extracts of metastatic mammary carcinomas | | + | Colcher et al. (1981) |
| B 6.2 | | | + | Stragmignoni et al. (1983) |
| B 1.1 | | | + | |
| Ca 1 | glycoprotein extract from H.Ep.2. cell line (laryngeal carcinomas) | | + | Ashall et al. (1982) |
| | | | + | McGee et al. (1982) |

N.B. This compilation does not pretend to be complete. Only those colorectal cancer reactive monoclonal antibodies, which were intensively studied or received much attention in the literature, are mentioned.

S indicates, that the monoclonal antibodies are reported to be colorectal carcinoma specific.

+ indicates, that the monoclonal antibodies were screened in the system indicated at the top of the column.

It is apparent, that most monoclonal antibodies have been screened on cell lines, but that immunocytochemical screening on tissue sections has been scarcely conducted. It should be noted, that the monoclonal antibodies claimed to be colorectal cancer specific have not been tested in tissue sections, so that their specificity remains questionable.

be concluded, that few monoclonal antibodies have been studied rigorously enough to substantiate the claim of colorectal cancer specificity.

1.5.2. Tissue-antigens

Tissue antigens may be divided into antigens on cell constituents and on cell products.

a. Cell constituents

Cytoplasmic antigens. The existence of intermediate filament proteins has been identified in alcohol fixed tissue of normal colonic mucosa and colorectal carcinomas (Altmannsberger et al., 1982). Electron microscopic studies have revealed a gradual disorganization of the three-dimensional network of intermediate filaments loosely surrounding the nucleus and inserting into lateral desmosomes in colonic tumours of increasing anaplasia (Brown et al., 1983). Since cytoskeletal antigens seem to be retained even in poorly differentiated carcinomas, their immunocytochemical demonstration may be of great value to type highly anaplastic tumours, which on morphological grounds may be difficult to classify (Altmannsberger et al., 1982).

Membrane antigens. Alterations in composition of membrane antigens, mainly of glycoprotein nature in colorectal adenocarcinomas as compared to normal colonic mucosa have been studied in respect of various antigens with different techniques. Blood group antigens of the A, B, H system have been detected in about half of rectosigmoid carcinomas by means of red cell adherence tests (Denk et al., 1974; Cooper and Haesler, 1978; Abdelfattah-Gad and Denk, 1980) and also with monoclonal antibodies direct against A and B antigens (unpublished observations, Arends et al., 1984). These antigens do not seem to occur in normal adult colonic mucosa, but can be observed in fetal large bowel mucosa (Szulman, 1964).

Also colorectal cancer reactive monoclonal antibodies detecting sugar sequences present on glycolipids of the Lewis blood group system have been described (Hounsell and Feizi, 1982; Magnani et al., 1982). Blood group antigens predominantly appear to show a patchy expression pattern in colorectal neoplasms (Cooper et al., 1980).

The same mode of expression has been reported for HLA-DR antigens, which are detectable by means of monoclonal antibodies on cell lines of colonic cancer (Pollack et al., 1981; MacLean et al., 1982) and on about half of the colorectal adenocarcinomas in spite of their absence in normal epithelium (Daar et al., 1982; Daar and Fabre, 1983). HLA-ABC antigens seem to be expressed in normal as well as neoplastic mucosa (Daar and Fabre, 1983).

Lectin affinity studies have been employed to explore the composition of glycoproteins on membranes and in the cytoplasm of colorectal cancer cells and the alteration of these antigens in relation to normal mucosa (Kim et al.,

1974; Boland et al., 1982; Paulie et al., 1983).

The epithelial membrane antigen (EMA), which is detected by an antiserum raised against human milk fat membrane globules, has been reported to occur in colorectal (cancer) tissue (Heyderman et al., 1979; Sloane and Ormerod, 1981). Also monoclonal antibodies generated against human milk fat membranes, like HMFG (Taylor-Papadimitriou et al., 1981) and mam 3 (Hilkens et al., 1982) stain colorectal mucosa and colonic neoplasms in a variable staining pattern.

b. Cell products

Normal crypt lining colonic epithelium mainly consists of columnar or absorptive cells and mucin producing goblet cells. In the base and deep regions of the crypts some enteroendocrine cells are present. In the normal situation Paneth cells are not seen, but these can occur in inflammatory and neoplastic conditions.

Goblet cell antigens. Many studies have been devoted to gastrointestinal mucins (for review see Filipe et al., 1979). According to conventional histochemical staining techniques colonic mucus appears to be a mixture of neutral, sialo-, and sulphomucins produced by the goblet cells of the epithelium. In normal colonic mucosa sulphomucins prevail over sialomucins, whereas neutral mucins are present in only moderate amounts. The composition of goblet cell mucins shows a regional variation over the various segments of the colorectum as well as alterations in relation with the position of the mucin producing cells along the crypt (Greco et al., 1967; Filipe, 1979). Colorectal adenocarcinomas generally demonstrate a predominance of sialomucins over sulphomucins (Filipe et al., 1979; Montero and Segura, 1980) and the same situation is observed in mucosa adjacent to these carcinomas, the so-called transitional mucosa (Filipe and Branfoot, 1974; Dawson and Filipe, 1976). Therefore a shift from predominance of sulphomucin production to increased sialomucin secretion in normal colonic mucosa has been taken to indicate a premalignant transformation, a notion which has become contested by the observation that the same changes of mucus production occur adjacent to other types of neoplasia and inflammatory conditions (Isaacson and Attwood, 1979; Franzin et al., 1983).

Several conventional and monoclonal antibodies appear to detect epitopes on mucin substances, which are often poorly biochemically characterized. Some conventional antibodies of this nature are listed below.

Conventional antibodies detecting mucosubstances

| <u>designation</u> | <u>abbreviation</u> | <u>literature</u> |
|---|---------------------|-------------------------------|
| Mucus-associated gastrointestinal antigen | M3 | Bara et al., 1977, - 1980a, b |

| | | |
|---|-------|--|
| Intestinal mucosa specific glycoprotein | IMG | Kawasaki et al., 1972, 1974 |
| Goblet cell antigen | GOA | Rapp et al., 1979 |
| Colonic Mucoprotein antigen | CMA | Gold and Miller, 1974, Gold, 1981 |
| Colon-specific-antigen-p | CSA-p | Pant et al., 1978; Shochat, et al., 1981 |
| "Another oncofetal antigen" | - | Ma et al., 1980 |

Columnar cell antigens. Secretory component (SC), a glycoprotein with a molecular weight of 60-70 kD has a carrier transport function in the epithelial translocation of dimeric IgA (Tourville et al., 1969; O'Daly et al., 1971; Brandtzaeg, 1974; Brown et al., 1976) and has been reported to occur in many epithelia involved in the so-called local secretory immune system (Tourville et al., 1969; Harris and South, 1981). In the large intestine SC is synthesized by the columnar cell population (O'Daly et al., 1971) and as such can be regarded as a differentiation antigen of this type of epithelium. Several reports have been published concerning its immunocytochemical expression in relation to the stage (Rognum et al., 1980; 1982) and histological grade (Poger et al., 1976; Weisz-Carrington et al., 1976; Green et al., 1977; Rognum et al., 1980, 1982; Isaacson, 1982) of colorectal carcinomas, which will be discussed in greater detail in chapter V of this study.

Enteroendocrine cell antigens. Neuroendocrine cells producing various peptide hormones have been described in the mucosa of the colon and especially the anorectum (Fenger and Lyon, 1982). Carcinoids (i.e. tumours of pure neuroendocrine cell composition) of hindgut origin often react with argyrophilic stains (Hosoda et al., 1975; Taxy et al., 1980; Fenger and Lyon, 1982;). Argyrophilic cells can also be observed in combined adenomatous and carcinoid tumours (adenocarcinoids) (Bates and Belter, 1967) and in ordinary well-differentiated adenocarcinomas (Ulich et al., 1983).

Expression of neuron-specific enolase (NSE), an enzyme, which is generally detected by immunocytochemical methods, has been noticed in most, if not all, cells showing neuroendocrine activity (Schmechel et al., 1978) and could therefore be of use in the study of carcinomas with such features of the gastrointestinal tract.

The peptide hormones, which have been detected in normal and neoplastic enteroendocrine cells of the rectosigmoid by immunocytochemical means include glucagon, somatostatin, gastrin, motilin, secretin, neurotensin, glicentin, pancreatic polypeptide, enkephalin and β -endorphin (Alumets

et al., 1980; Ulich et al., 1983; Fiocca et al., 1980; O'Brian et al., 1982; Yang et al., 1983; Alumets et al., 1981).

Paneth cell antigens. Lysozyme, an enzyme capable of lysis of bacterial cell walls (Chipman and Sharon, 1969; Hill and Porter, 1974) is a product of Paneth cells (Ghoos and Vantrappen, 1971; Peeters and Vantrappen, 1975; ; Klockars and Reitamo, 1975). In normal colonic mucosa immunoreactivity for lysozyme is absent (Mason and Taylor, 1975; Kittas et al., 1982), but immunoreactive cells can be seen in inflamed epithelium (Watson and Roy, 1960) and mucosa adjacent to carcinomas (Symonds, 1974). In general the enzyme is not expressed in colorectal carcinomas with exception of metaplastic Paneth cells which may occur in these tumours (Holmes, 1965; Gibbs, 1967), but the colloid variety demonstrates strong cytoplasmic immunoreactivity for lysozyme, often in combination with neuroendocrine features (Arends and Bosman, 1983).

1.6. Antigen-specificity in colorectal carcinomas

In the previous paragraph we have described the most relevant antigens in colorectal carcinomas. We will now address the problem of their specificity more explicitly in view of the fact that the identification of an antigen occurring exclusively on colorectal (tumour) cells and not on other neoplastic or normal adult tissues would be of tremendous help for the characterization and classification of colorectal carcinomas as well as the demonstration of metastatic lesions as of colorectal origin (vide 1.2, sub a). Antigen-specificity may be related to the unique expression of an antigen in a special type of tumour or to its exclusive occurrence in normal tissues of a given histological type and in their derivative neoplasms.

As indicated in 1.5.1. monoclonal antibody-defined antigens, originally believed to be colorectal cancer-specific, fail to meet the claim of specificity after more extensive study and other antigens reported to be of such nature have not yet been explored rigorously enough to substantiate their hailment as "colorectal-specific". As a result at this moment no tumour-specific antigens have been conclusively established in colorectal carcinomas.

Likewise, tissue-antigens specific for colorectal epithelial cells and their derivative tumours as yet have not been convincingly demonstrated. Cellular constituents, such as intermediate filaments and the majority of membrane glycoproteins also occur in other histological tissue types and the same situation applies to cellular products such as mucins, SC, peptide hormones and lysozyme.

Therefore, attempts to type colorectal carcinomas and to identify metastatic lesions as of colorectal origin are frustrated by the lack of specificity of the available antigens and as yet remain unsatisfactory.

However, despite the apparent lack of specificity of the antigens studied in connection with colorectal carcinomas determination of their expression may still be of use to classify colorectal tumours according to their parental cell type, differentiation or stage of maturation.

1.7. Phenotypic heterogeneity of antigen expression

The recognition that tumours are not uniform entities consisting of cells with similar properties, but are highly heterogeneous and contain multiple subpopulations of cells with different properties, is steadily increasing (see for excellent reviews Fidler and Hart, 1982; Poste, 1982; Woodruff, 1983). First of all, neoplasms contain normal cells (inflammatory cells, blood vessels), transformed cells, cells conceivably having taken some but not all necessary steps in the development towards malignancy (initiated cells) and possibly also hybrid cells (created by fusion of two cells of the same or different categories). Furthermore, some neoplastic cells are so-called clonogenic cells, which are responsible for infiltrative growth and metastasis of neoplasms.

Obviously, these clonogenic cells are the main targets of research concerning the biological behaviour and manipulation of neoplastic growth and these cells have also been shown to be heterogeneous in respect of their DNA content (Peterson et al., 1978; Vindelov et al., 1980), growth rate (Butel et al., 1977) antigenic composition (Prehn, 1970; Rutzky and Siciliano, 1982) metastatic potential (Brattain et al., 1980; Spremulli et al., 1983) and response to cytotoxic drugs (Barranco et al., 1973).

The implications of the phenomenon of phenotypic heterogeneity of antigen expression in respect of the potential clinicopathological value of determination of antigenic display, as outlined in 1.2. a and b, are apparent. Heterogeneity of antigen expression may limit the potential value of determination of antigenic composition in regard of the characterization and classification of primary tumours and metastases of unknown origin (vide 1.2. sub a). The typing of primary tumours may become impossible, when an area of a given tumour is studied, which happens to be devoid of the antigen under investigation. An even more important problem, however, may arise in the typing of metastatic tumours. Since experimental data indicate, that tumours consist of subclones of tumour cells with a widely differing metastatic potential, it is conceivable, that as a result of clonal selection during the evolution of metastases the antigenic composition of neoplastic cells in metastatic tumours may be more restricted than in the parental tumours (Fidler, 1978). The existence of such a phenomenon would clearly thwart the identification of metastatic lesions according to their antigenic display and also would gravely interfere with immunotargeting procedures, which especially,

when applied with therapeutic options, are meant to reach as many tumour cells as possible.

Phenotypic heterogeneity of antigen expression in tumours in addition may form an important obstacle in procedures aimed at the assessment of the grade of malignancy (vide 1.2. sub b). These procedures generally rely on the establishment of a correlation between antigen expression and other clinico-pathological variables of known prognostic significance. Many of these variables demonstrate heterogeneity per se, as e.g. histological grade (Qualheim and Gall, 1953), which in addition to stage of tumour extension, is known to be an important prognostic index in colorectal tumours. In the simple over-all classification of antigen expression into categories of "positive", "focally positive" or "negative" and histological grade into well-, moderately- and poorly differentiated, which for practical purposes are commonly performed, the intercellular heterogeneity of both parameters is not taken into account. Thus correlations which might exist between these parameters at the individual cell level might be obscured. Also, apart from these practical considerations, by the virtue of simple immunocytochemical demonstration the significance of an antigen heterogeneously expressed in one or more subclones of a neoplasm may be impossible to assess in terms of the biological behaviour of the tumour as a whole.

In view of this situation it may not be surprising that the expression of blood group antigens of the A, B, H type and antigens of the HLA systems, which often show a focal expression pattern do not appear to be related with the histological grade of colorectal carcinomas (Abdelfattah-Gad and Denk, 1980). Also the prognostic significance of mucus production by colorectal carcinomas remains controversial (Trimpi and Bacon, 1951; Wolman et al., 1957; Symonds and Vickery, 1976; Pihl et al., 1980), probably due to improper delineation of the type and the extent of mucin expression by these tumours.

Since several reports indicate a correlation between the expression of SC and histological grade (Poger et al., 1976; Weisz-Carrington et al., 1976; Green et al., 1977; Rognum et al., 1980, 1982; Isaacson, 1982) as well as stage (Rognum et al., 1980, 1982) of colorectal carcinomas, this antigen so far appears to be one of the few with a potential prognostic value.

1.8. Design of the study

From the outline given in the previous paragraphs based on a review of the literature it may be apparent that the demonstration of tumour-antigen expression may have its potential value in regard of the characterization and classification of primary tumours and metastases as well as the assessment of the grade of malignancy but at the same time is subject to limitations posed by the problems of antigen-

specificity and phenotypic heterogeneity of antigen expression.

The present study addresses these topics of apparent relevance to the study of tissue antigen expression in connection with colorectal carcinomas.

Chapter II deals with the problem of antigen-specificity in respect of the monoclonal antibody 1116 NS 19-9 defined monosialoganglioside or gastrointestinal cancer associated antigen (GICA), because of its original hailment as an oncofetal, non CEA related antigen occurring on some gastrointestinal cancers, but not on normal adult tissues. In chapters III, IV and V the potential values in respect of prognosis are evaluated in regard of GICA and SC.

The topic of heterogeneity of antigen expression is addressed in Chapter VI in connection with the extent of phenotypic heterogeneity of antigen expression in primary colorectal tumours and possible differences in antigen profile between primary tumours and their regional lymphnode as well as hematogenous metastases. At the same time the interference of antigen heterogeneity in primary and metastatic tumours with the efficacy of immunotargeting procedures has been considered.

Chapter VII, the epilogue, summarizes the results of the study and evaluates the present limitations and future perspectives of tissue detection of tumour-antigens in colorectal cancer in the context of the data obtained.

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Chapter II

DISTRIBUTION OF MONOCLONAL ANTIBODY-DEFINED MONOSIALOGLANGIOSIDE IN NORMAL AND CANCEROUS HUMAN TISSUES: AN IMMUNOPEROXIDASE STUDY

J. W. Arends , C. Verstijnen , F. T. Bosman , J. Hilgers
and Z. Steplewski .

2.1. Summary

The immunoreactivity of a monosialoganglioside antigen defined by monoclonal antibody 1116NS19-9 (19-9) was studied in neoplastic and normal glandular and mucosal epithelia using an indirect immunoperoxidase method. In neoplastic mucosae, the antigen was detected in the majority of colorectal and endometrial carcinomas, predominantly in a focal staining pattern. A substantial proportion of gastric and pancreatic tumours and an occasional breast carcinoma also reacted with the monoclonal antibody. Expression of the monosialoganglioside in normal colonic mucosa appeared to be restricted to areas adjacent to tumour tissue. In gastric mucosa the antigen was confined to some areas showing intestinal metaplasia. The antigen was also detected in the epithelium of normal mucosa of the gall bladder and endocervix, as well as in some ductal epithelia of the pancreas and salivary glands. Most other mucosae were negative for antigen expression.

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2.2. Introduction

Monoclonal antibodies against colon carcinoma were first reported by Koprowski et al. (1979). The binding to colon carcinoma cells of one of these monoclonal antibodies was inhibited by sera from patients with adenocarcinoma of the colon, stomach, and pancreas, but not by sera from patients with other bowel diseases or from healthy individuals (Koprowski et al., 1981). Furthermore, the antibody did not react with carcinoma cell lines other than colorectal (Koprowski et al., 1979). The target antigen was subsequently defined as a monosialoganglioside (Magnani et al., 1981), which was demonstrable in colorectal carcinoma tissue and meconium only. Because the antigen appeared to retain sufficient immunoreactivity after routine fixation and tissue processing procedures, it became possible to determine its distribution and precise tissue localization in different organs by means of an immunoperoxidase technique.

We report here the results of the immunoreactivity assays of this monoclonal antibody-defined monosialoganglioside in adenocarcinoma and in fetal and adult glandular tissues.

The antigen is not restricted to oncofetal tissues, as it can be detected in normal adult epithelia of gall bladder, endocervix, pancreas, salivary glands, and colon. Most colonic adenocarcinomas and other adenocarcinomas of the endometrium, pancreas, and stomach demonstrated the antigen. Staining of normal colonic mucosa was restricted to areas adjacent to tumour tissue.

2.3. Material and methods

2.3.1. Monoclonal antibody

The hybridoma 1116NS 19-9 (19-9), which secretes an antibody against the SW1116 colon carcinoma cell line, was established after fusion of immune splenocytes with the 653 variant of P3X63Ag8 myeloma cells.

2.3.2. Tissues

The immunoreactivity of the 19-9 antibody was tested on paraffin sections, fixed in 4% neutral buffered formalin of the following human tissues: a panel of adenocarcinomas (Table 1), a total of 17 fetal intestinal mucosa from 12-40 week fetuses (kindly provided by Dr. J. Huber, Professor of Pediatric Pathology, State University, Utrecht, The Netherlands); and normal adult glandular and mucosal epithelia (Table 2).

2.3.3. Immunocytochemistry

Initially, to determine the influence of routine tissue processing procedures on the immunoreactivity of the antigen, the staining patterns of frozen sections postfixed in

acetone (10 min at 4 C) and of formalin fixed and paraffin-embedded tissue sections of the same tumour were compared. Paraffin-embedded tissue sections (5 m) were then deparaffinized, rehydrated, and blocked for endogenous peroxidase activity by incubation in a 0.5% solution of hydrogen peroxide in methanol. After incubation in a 0.1% trypsin solution (Sigma Chemical Company) in 0.1% CaCl_2 (pH 7.8) for 25 min at 37 C, sections were incubated with normal rabbit serum (1:5) for 10 min and then with 19-9 monoclonal antibody for 30 min. The optimal antibody dilution for paraffin sections was found to be 1:256; the antibody was used undiluted for frozen sections. Sections were then exposed to peroxidase-labeled rabbit anti-mouse Ig (DAKO, code no. P161) (1:50) for 30 min. All dilutions were made with 2.5% normal human serum in Tris buffer (pH 7.6). Each step was followed by washing in Tris buffer (three changes in 5 min). Finally the sections were developed with diaminobenzidine and counterstained with hematoxylin.

2.4. Results

The distribution of the monoclonal antibody-defined monosialoganglioside did not appear to be different on mildly fixed frozen as compared with formalin-fixed, paraffin embedded sections of the same tissues, when the antibody was applied undiluted on the former and used after trypsinization on the latter. When diluted monoclonal antibody was applied on frozen sections or undiluted antibody was used on nontrypsinized paraffin sections, no consistent pattern of staining was obtained.

2.4.1. Adenocarcinomas

Thirty-eight of 54 (70%) of colorectal adenocarcinomas of the colorectum and eight of 11 (73%) endometrial adenocarcinomas reacted with 19-9 antibody. Seven of 13 (54%) gastric and two of three pancreatic adenocarcinomas also reacted with antibody 19-9 (Table 1). One of two gall bladder carcinomas and only one of 10 breast carcinomas were reactive. The two carcinomas of prostate and two of kidney tested were negative for the presence of the antigen. The majority of adenocarcinomas of the colorectum (Fig. 1) and endometrium (Fig. 2) reacted with the 19-9 antibody, predominantly as a focal staining of apical cell borders and secretion products in glandular tumour formations. In some tumours, cytoplasmic dots were occasionally observed. Adenocarcinomas of the pancreas (Fig. 3), stomach (Fig. 4), and gall bladder, as well as one case of breast carcinoma (Fig. 5) showed a focal reaction product in glandular formations.

Table 1. Distribution of 19-9 antibody binding to adenocarcinomas

| | No. of positive cases / Total no. of cases tested | % positive | Pattern of reactivity |
|--------------|---|---------------|--|
| Colorectum | 38 / 54 | 70 | Apical cell parts and luminal debris predominantly in a fo- cal pattern. |
| Stomach | 7 / 13 | 54 | Apical cell parts, cytoplasm of signet ring cells and mucus. |
| Pancreas | 2 / 3 | 66 | Luminal cell surface of cyto- plasm of tumour cells. |
| Gall bladder | 1 / 2 | 50 | Cytoplasm of tumour cells. |
| Endometrium | 8 / 11 | 73 | Apical cell parts. |
| Kidney | 0 / 2 | 0 | — |
| Prostate | 0 / 2 | 0 | — |
| Breast | 1 / 10 | 10 | — |

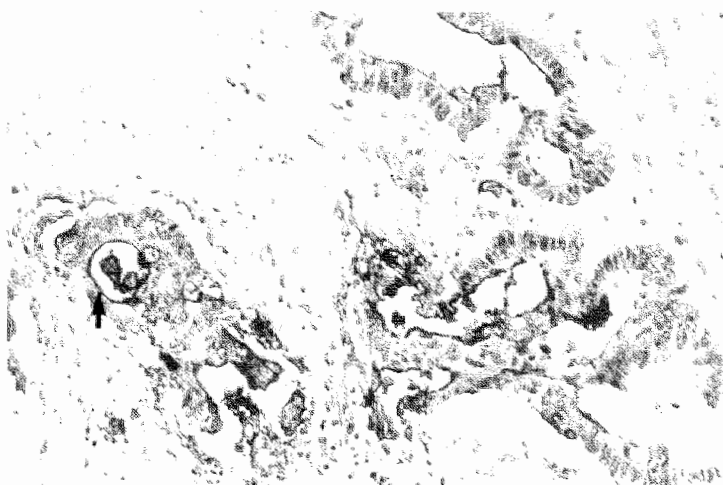


Fig. 1. Colonic adenocarcinoma, a reaction product at the luminal cell border (arrow), x 125.

2.4.2. Adult glandular and mucosal epithelial

The results of the immunoperoxidase assay for the detection of the antibody 19-9 defined antigen in adult glandular and mucosal epithelia are summarized in Table 2. Twelve of 20 (60%) colorectal mucosa specimens and five of 10 (50%) gastric mucosa specimens showed positive staining with the 19-9 antibody only in areas adjacent to the tumour. Endocervical mucosa and salivary gland ductal epithelium were also positive, and in three of four pancreas specimens ductal epithelium and in six of seven gall bladder specimens mucosal epithelium also showed staining. Normal kidney, prostate, and breast were all negative for the antigen. Normal large intestinal mucosa showed no staining. Immunoreactivity detected in normal colonic epithelium in cancer containing specimens was confined to areas adjacent to the tumour, and appeared either as weak staining of the apical cell surface (Fig. 4A) or as strong cytoplasmic staining of some goblet cells in the crypt lining epithelium (Fig. 4B). Breast, prostate, and renal cortex epithelia did not show immunoreactivity with the antibody. Gastric epithelium only stained in areas of intestinal metaplasia or in the immediate vicinity of a tumour. Columnar cells in endocervical mucosa (Fig. 5), salivary gland ductal epithelium (Fig. 6), and pancreas showed marked immunoreactivity, particularly the gall bladder epithelium, which consistently showed intense staining (Fig. 7). A focal staining pattern was generally observed in these tissues.

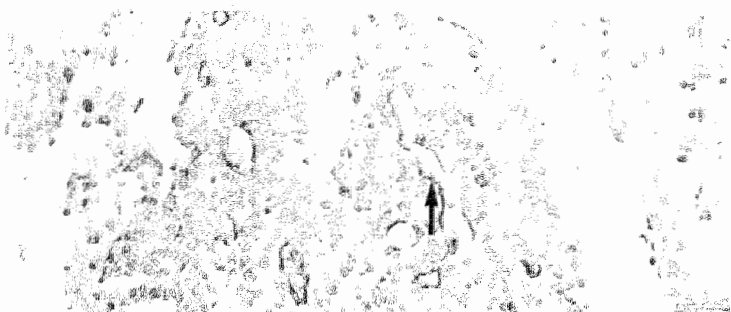


Fig. 2. Adenocarcinoma of endometrium, x 200.

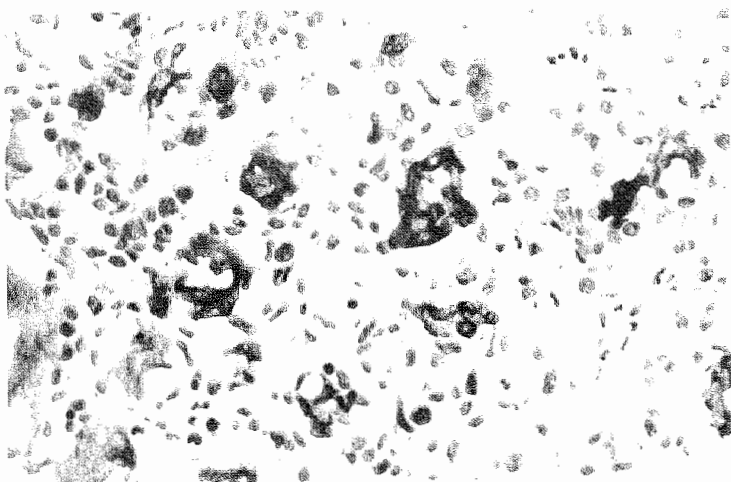


Fig. 3. Adenocarcinoma of pancreas, x 200.

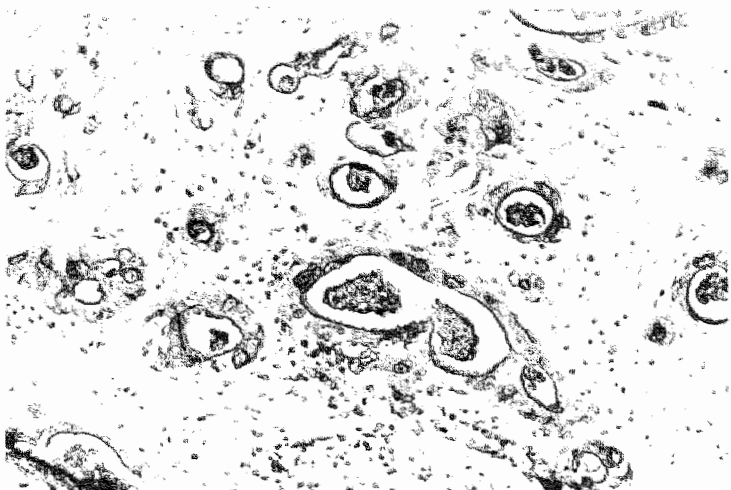


Fig. 4. Adenocarcinoma of stomach, x 125.

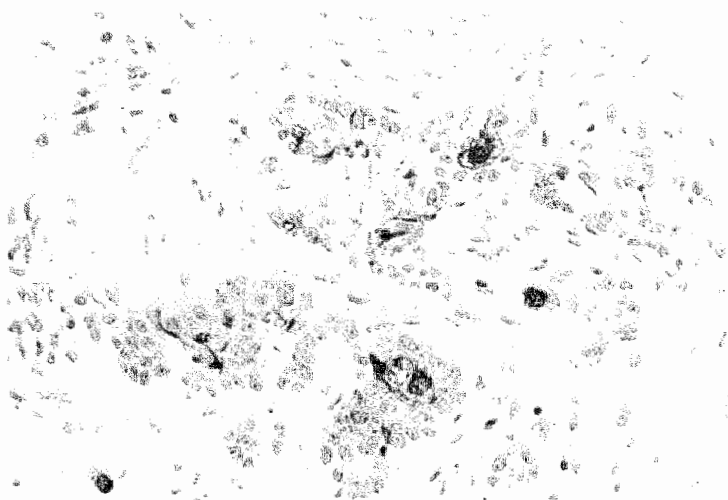


Fig. 5. Adenocarcinoma of breast, x 200.

Table 2. Distribution of 19-9 antibody binding to various adult glandular and mucosal epithelia.

| | No. of positive cases / Total no. of cases tested | % positive | Pattern of reactivity |
|---------------------|---|---------------|---|
| Colorectal mucosa | 12 / 20 | 60 | Surface of crypt lining cells. Cytoplasm of some goblet cells. |
| Gastric mucosa | 5 / 10 | 50 | Surface of glandular epithelium in the vicinity of tumour |
| Pancreas | 3 / 4 | 75 | Luminal cell border of the ductal epithelium. |
| Salivary gland | 2 / 2 | 100 | Luminal cell border of the ductal epithelium. |
| Gall bladder | 6 / 7 | 86 | Luminal cell surface and cyto- plasm of the columnar epithe- lium. |
| Endocervical mucosa | 9 / 9 | 100 | Cytoplasm of the columnar epi- thelium in a focal distribu- tion pattern. |

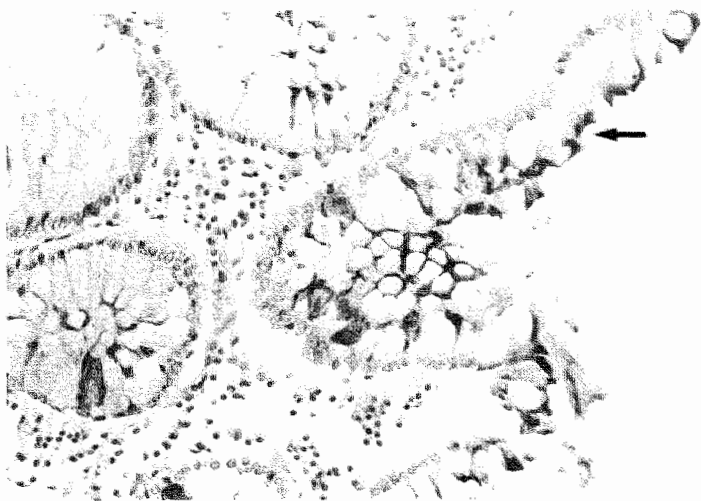


Fig. 6. Colonic mucosa adjacent to adenocarcinoma. A staining of the apical cell border is to be seen, x 200.

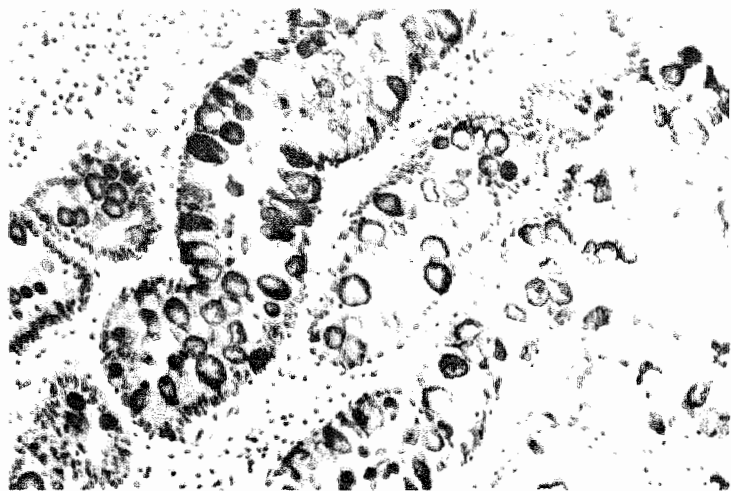


Fig. 7. Colonic mucosa adjacent to adenocarcinoma. In this case multiple reactive goblet cells are observed, x 200.

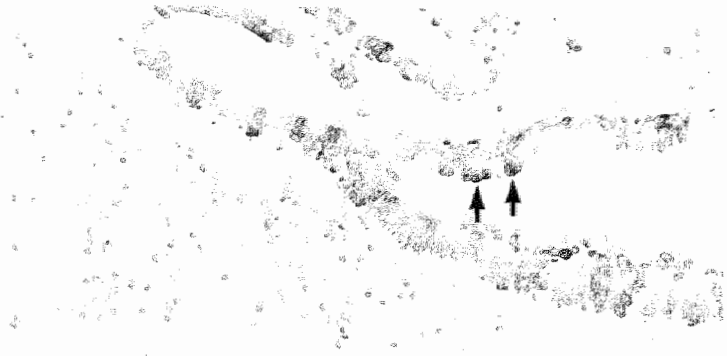


Fig. 8. Normal endocervical glandular mucosa. Note the focal distribution pattern and a cytoplasmic staining reaction (arrow), x 200.

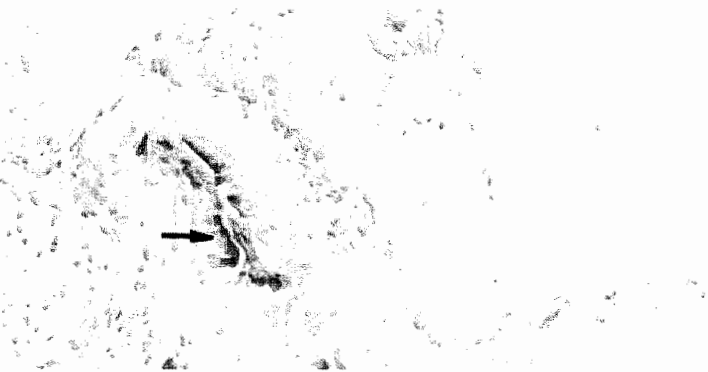


Fig. 9. Duct of normal salivary gland with a reaction product at the luminal cell border of the lining epithelium (arrow), x 125.

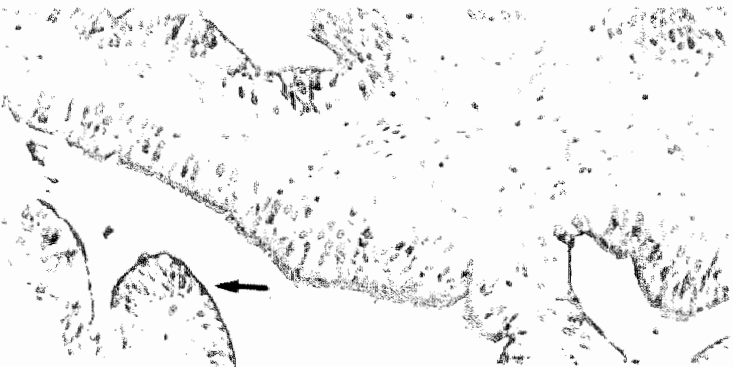


Fig. 10. Normal mucosa of gall bladder with a staining of the apical cell border, x 200.

2.4.3. Fetal intestinal tissue

Goblet cells of intestinal mucosa of 12-week embryos were positive for the 19-9 defined antigen, showing a moderate staining intensity (Fig. 8A). In later stages of gestation, the antigen was also expressed on the brush border of the columnar epithelium in the small and large bowel (Fig. 8b).

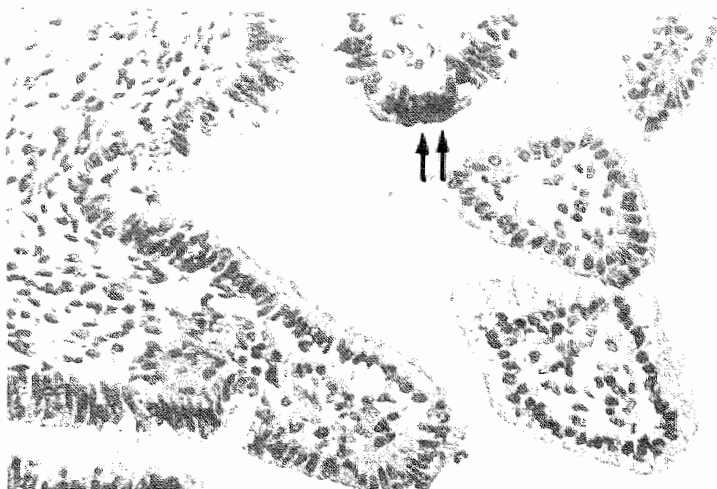


Fig. 11. Fetal intestinal mucosa of 12 wks. Note a few moderately stained goblet cells (arrow) in the superficial mucosa, x 200.

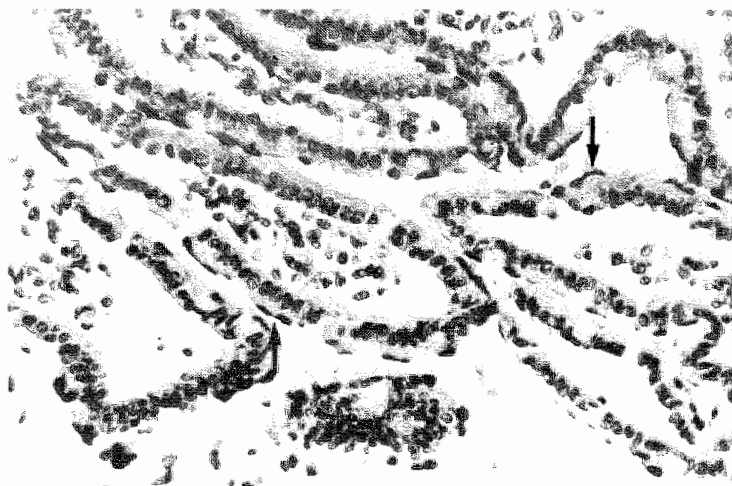


Fig. 12. Fetal intestinal mucosa of 22 wks. The reaction product is located at the luminal cell surface (arrows), x 125.

2.5. Discussion

The 19-9 monoclonal antibody against colonic carcinoma was originally described as reactive only with colorectal carcinoma cells, and not with normal colonic epithelium or with carcinomas other than of the colon (Koprowski et al., 1979). It was also demonstrated that binding of this antibody to SW1116 colon carcinoma cells was inhibited by sera from patients with adenocarcinoma of the colon, stomach, and pancreas, but not by sera from patients with non-neoplastic intestinal disease or from healthy individuals (Koprowski et al., 1981; Herlyn et al., 1982). Finally, the target antigen was identified as a monosialoganglioside, which could be detected in colorectal carcinoma tissue and meconium only (Magnani et al., 1981) and which showed no immunologic similarity to carcinoembryonic antigen (CEA).

These experimental data suggested that the antigen might be oncofetal and potentially useful for the detection of colorectal carcinoma. We decided to perform a systematic survey on the distribution of the antigen in various tissues, using an immunoperoxidase method. Tissue sections rather than cultured cells were chosen, because they play a substantial role in the selection and characterization of monoclonal antibodies with a desired specificity (Finan et al., 1982). The results indicate that the target antigen is not oncofetal, since it was detected in differentiated normal adult glandular epithelia, such as endocervix, gall bladder, pancreas, and salivary gland. The distribution of the antigen in mildly fixed frozen sections closely paralleled that in trypsin treated sections, excluding the possibility that antigenic properties were altered by routine tissue processing methods. Thus the discrepancies between results of tests on cell lines and on tissue sections cannot be explained by differences in the techniques used. The antigen, however, does appear to be present abundantly in most colorectal and endometrial adenocarcinomas, whereas it is absent or almost absent in normal mucosae as shown also by Atkinson et al., (1982). In adult colonic mucosa the antigen appears to be localized only in areas adjacent to tumour tissue, either weakly staining the luminal surface of crypt lining cells, which could be the result of shedding of antigenic material into the surrounding tissue (Koprowski et al., 1981), or strongly staining the cytoplasm of a few Goblet cells. The intense staining of the latter cells suggested that they might produce the antigen. In individuals with positive Le phenotype (Magnani et al., 1982; Koprowski et al., 1982; Steplewski et al., 1983) the antigen would be expected to be represented in the salivary glands and their secretions since this particular monosialoganglioside represents a sialylated Le hapten (Magnani et al., 1982; Koprowski et al., 1982; Steplewski et al., 1983; Falk et al., 1983).

The 19-9 target antigen appears to be more widely distributed than originally proposed. It is conceivable, how-

ever, that it is present in such small quantities in tumour adjacent epithelium that it can only be detected using a very sensitive immunoperoxidase procedure. Furthermore, cultures of cancer cells probably represent only a limited selection of the cells present in the original tumour and, in addition, may lose some characteristics of the original tumour during cultivation. For these reasons immunohistochemical studies of cancerous tissues should be considered an essential step in the final characterization of antitumour monoclonal antibodies.

The question remains whether the presence of the antigen in the vicinity of a tumour indicates a premalignant alteration of normal mucosal epithelium (Filipe, 1979) or a reaction to adjacent malignant cell proliferation (Isaacson and Attwood, 1979).

It is conceivable that the presence or absence of the 19-9 target antigen is related to the level of differentiation of colonic adenocarcinoma. This question is currently under investigation.

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Chapter III

MONOCLONAL ANTIBODY (1116 NS 19-9) DEFINED MONOSIALOGLANGLIOSIDE (GICA) IN COLORECTAL CARCINOMA IN RELATION TO STAGE, HISTOPATHOLOGY AND DNA FLOW CYTOMETRY.

J.W. Arends , T. Wiggers , B. Schutte , C.T. Thijs , C. Verstijnen , J. Hilgers , G.H. Blijham and F.T. Bosman .

3.1. Summary

Immunoreactivity of 1116 NS 19-9 monoclonal antibody defined monosialoganglioside (gastrointestinal cancer associated antigen, GICA) has been studied in a series of colorectal carcinoma patients of a multicentre prospective controlled trial in order to assess its correlation with parameters such as localization, stage, histopathological characteristics and DNA flowcytometry. GICA could be detected in 60% of the carcinomas, but no correlation was observed between its status of immunoreactivity and any of the parameters studied.

It is concluded, that, though study of the expression of the monosialoganglioside may be worthwhile in relation to fundamental aspects of behaviour of colorectal carcinomas, the significance of its immunohistochemical detection in a diagnostic or prognostic sense is limited.

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3.2. Introduction

In 1979 Koprowski and co-workers were the first to establish a large series of stable hybridoma clones secreting colorectal carcinoma - reactive monoclonal antibodies (Koprowski et al, 1979). One of these antibodies, produced by the 1116 NS 19-9 clone, appeared to possess promising characteristics, since it did not react with carcinoma cell lines other than colorectal. Binding of this antibody was inhibited by serum from patients with adenocarcinoma of the colon, stomach and pancreas, but not by serum from patients with other forms of intestinal pathology or from healthy individuals (Koprowski et al, 1981). Furthermore, the target-antigen was found in sera and urine of colon carcinoma patients (Chang et al, 1981).

The target-antigen was characterized as a monosialoganglioside, which was claimed to be detectable in colorectal tissue and meconium only (Magnani et al, 1981). Based on these findings the antigen was considered to be of oncofetal nature and potentially useful for the detection and study of colorectal carcinomas (Steplewski and Koprowski, 1982) and a kit (Centocor Ca 19-9 TM RIA) for assessment of antigen levels in serum and urine was marketed for use in gastrointestinal cancer.

However, immunoperoxidase studies on the monoclonal-antibody defined monosialoganglioside (Gastrointestinal Cancer - associated antigen, GICA) have shown it to be more widely distributed than originally proposed (Atkinson et al, 1982) and the oncofetal nature of the antigen has been challenged (Arends et al, 1983).

To further delineate the characteristics of the monosialoganglioside and to evaluate its usefulness as a marker in colorectal carcinoma we tested its immunoreactivity in histological sections of a large series of carcinomas of the large bowel and compared the results with gross and histological characteristics and with flow-cytometrical data.

The results indicate that the presence of GICA is neither correlated with the localization nor with the stage of the tumour. In addition, there appears to be no statistically significant correlation with the histological degree of differentiation of colorectal cancers, nor with DNA flow-cytometrical parameters.

3.3. Materials and methods

3.3.1. Tissues

Blocks of paraplast embedded colorectal carcinoma tissue were collected from a multicentre prospective controlled trial (carried out from jan. 1979 through jan. 1982), which compares the value of the conventional versus no-touch surgical approach in colorectal cancer. Patients with adjacent organ invasion or distant metastases were excluded from the

clinical trial, but this category of patients was included in this study, bringing the total number of cases to between 307 and 312, depending on the parameter studied.

3.3.2. Classification of specimens

All pathological specimens were reviewed by two independent observers (J.W. A. and C.T. T.) and classified according to their localization, stage and degree of differentiation.

3.3.3. Localization

Concerning localization, tumours of the cecum and colon ascendens were taken together, whereas tumours of the hepatic and splenic flexure were classified as carcinomas of colon transversum.

3.3.4. Staging

For staging purposes the Turnbull modification of the original Dukes' classification (Turnbull et al., 1967) was employed:

Stage A Tumour confined to the bowel wall.

Stage B Extension of tumour into pericolic fat.

Stage C Tumour with regional lymphnode metastases.

Stage D Distant organ metastases or infiltrative growth into adjacent organs.

3.3.5. Grading

The degree of differentiation was rated according to criteria adopted from Blenkinsopp et al., (1981) modified as follows:

- a. well-differentiated : tumours entirely consisting of glandular formations with up to two layers of lining cells with preserved nuclear polarity (fig. 1.A)
- b. poorly differentiated : tumours with more than 10% solid growth pattern (fig. 1.B)
- c. moderately differentiated : all tumours covering the spectrum between a. and b. (fig. 1.C-D).

Using these criteria the majority of tumours were graded as moderately differentiated. This group was further subdivided into "moderately differentiated" and "moderately differentiated tending to poor" (mod. poor). The latter category consisted of carcinomas with a tendency to form solid areas (up to 10%) or a marked cribriform growth pattern. (fig. 1.D). Tumours were classified as undifferentiated when they did not show any glandular structures. Mucinous carcinomas were graded as outlined above. Pure signetring-cell carcinomas were excluded from this study. Grading was based on examination of two or more sections from different parts of the tumour and the least differentiated part observed was selected for grading.

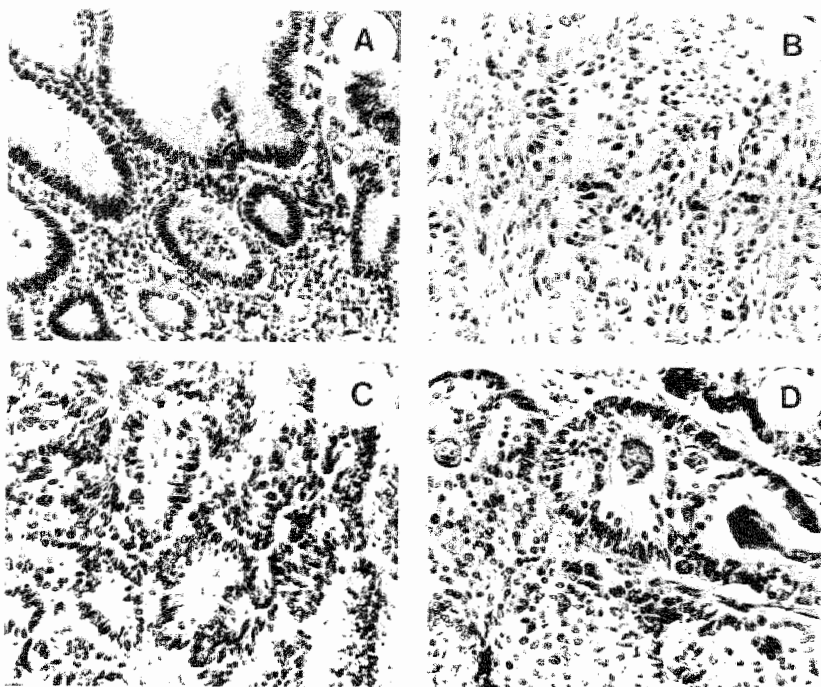


Fig. 1. A. Well-differentiated, the process is entirely made up of glandular structures. B. Poorly-differentiated, the tumour forms extensive solid areas. C. Moderately-differentiated, the epithelium of the glandular structures consists of more than two layers and nuclear polarity is not preserved. D. Moderately-differentiated with a tendency to poor differentiation (mod. poor), the tumour tends to grow in a cribriform or solid pattern. H/E x 150.

3.3.6. Immunohistochemistry

For immunohistochemical detection of the l116 NS 19-9 target antigen one block, preferably containing tumour tissue and adjacent normal mucosa, was studied. Details of the staining procedure, which was applied after trypsinization of the sections and using an indirect immunoperoxidase technique, have been published previously (Arends et al, 1983)

Staining with a non-immune mouse serum served as negative control and a known positive colorectal carcinoma was used as positive reference tissue.

3.3.7. Scoring of immunoreactivity

Immunoreactivity was scored according to distribution in such a way, that a distinction was made between tumours displaying a reaction product in over 80% of the areas of cancerous growth (positive), tumours reacting scarcely (less than 5% of all areas or not at all (negative)) and tumours showing reactivity in 5-80% of the neoplastic tissue, other parts being negative (focally positive) (fig. 2.A-B).

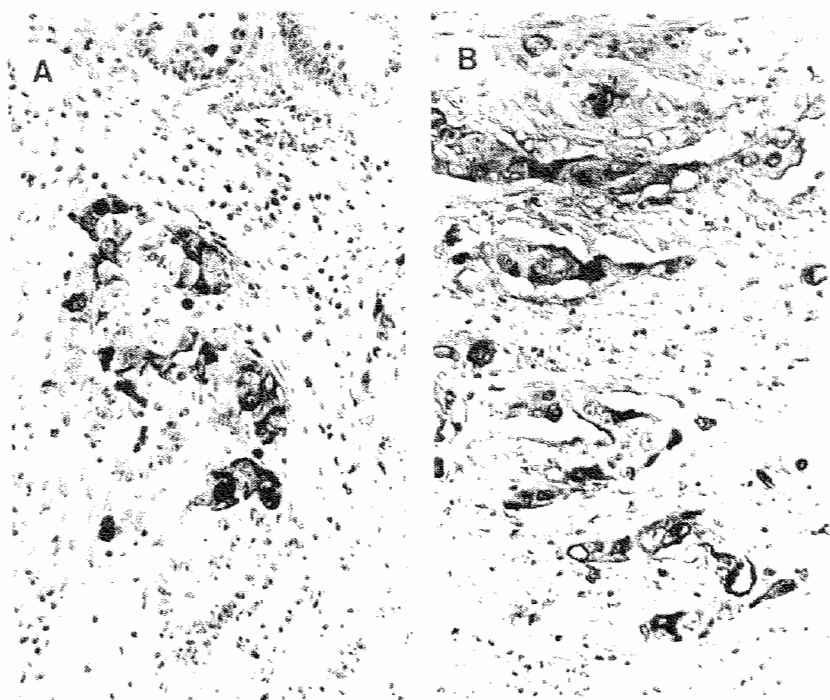


Fig. 2. Mode of GICA expression in coloncarcinoma: A. focal expression pattern. I.P. x 120. B. positive expression pattern. I.P.150.

3.3.8. Flowcytometry

Method: flow cytometric analysis was performed on isolated nuclei according to the method of Vindelov et al. (Vindelov et al, 1983a; Vindelov et al, 1983b). Briefly, fresh tumour tissue was minced with scalpels in a citrate buffer. Aliquots of 100 μ l (approximately 2.10^6 cells) were mixed with 900 μ l of solution A (trypsin 30 μ g/ml). After 10 minutes at room temperature 750 μ l of solution B (soybean trypsin inhibitor 500 μ g/ml, ribonuclease 100 μ g/ml) was added. After another 10 minutes at room temperature 750 μ l of ice cold solution C (propidiumiodide 42 μ g/ml, spermine-tetrahydrochloride 3 mM) was added. After each addition the tubes were inverted to mix the contents gently. Samples were kept on ice and run on a FACS IV (Becton & Dickinson, Sunnyvale, CA) between 15 minutes and 3 hours after staining. Nuclear DNA content was measured using chicken and trout red blood cells as reference standards and expressed as DNA index, i.e. the ratio of aneuploid G 1/0 peak channel and diploid G 1/0 peak channel. When possible the percentage of cells in S phase was calculated.

3.4. Results

3.4.1. GICA immunoreactivity in colorectal carcinoma

Of 312 cases of colorectal carcinoma 10,6% showed GICA immunoreactivity, 53,5% showed focal immunoreactivity and 35,9% was negative. In the positive and focally positive cases the reaction product was always localized in the apical cytoplasm and luminal secretion products (fig. 2.A-B). Immunoreactivity of normal adjacent colonic mucosa was studied in 250 cases. 39,6% was positive, invariably focal. In 7,2% the mucosa was positive, whereas the adjacent tumour was negative.

3.4.2. GICA expression and localization

The pattern of GICA immunoreactivity in relation to the site of the primary tumour is shown in table I. In none of the regions the percentage of positive tumours differed significantly from the overall percentage of immunoreactive tumours ($p = 0.2$).

TABLE I

GICA - IMMUNOREACTIVITY AND LOCALISATION OF THE TUMOUR

| | negative number/ (perc.) | focally positive number (perc.) | positive number/ (perc.) | total |
|---------------------------------|--------------------------------|--|--------------------------------|-------|
| Ascending colon | 22 (32.8) | 40 (59.7) | 5 (7.5) | 67 |
| Transverse colon | 11 (32.4) | 16 (47.1) | 7 (20.6) | 34 |
| Descending colon and sigmoid | 43 (43.4) | 48 (48.5) | 8 (8.1) | 99 |
| Rectum | 36 (32.4) | 62 (55.9) | 13 (11.7) | 111 |
| Total | 112 (36.0) | 166 (53.4) | 33 (10.6) | 311 |

$p = 0.2$

3.4.3. GICA expression and staging

In table II GICA immunoreactivity is shown relative to staging. Again no correlation between the two parameters was found ($p = 0.3$).

TABLE II

GICA - IMMUNOREACTIVITY AND STAGING

| | negative number/ (perc.) | focally positive number/ (perc.) | positive number/ (perc.) | total number |
|---------|--------------------------------|---|--------------------------------|-----------------|
| Stage A | 31 (44.3) | 35 (50.0) | 4 (5.7) | 70 |
| Stage B | 41 (34.5) | 64 (53.8) | 14 (11.8) | 119 |
| Stage C | 33 (35.1) | 48 (51.1) | 13 (13.8) | 94 |
| Stage D | 7 (24.1) | 20 (69.0) | 2 (6.9) | 29 |
| Total | 112 (35.9) | 167 (53.5) | 33 (10.6) | 312 |
| | | | $p = 0.3$ | |

3.4.4. GICA expression and grading

In table III GICA immunoreactivity is compared with tumour grade. No relation was found between GICA immunoreactivity and histological degree of differentiation. However, positive tumours did not seem to occur in the poorly differentiated and undifferentiated group and occurred slightly more often in the well differentiated and moderately differentiated tumours, whereas negative tumours tended to occur slightly more often in the group of poorly and undifferentiated carcinomas. This trend, however, did not reach statistical significance ($p = 0.5$).

TABLE III

GICA - IMMUNOREACTIVITY AND DIFFERENTIATION GRADE

| | negative | focally positive | positive | total |
|------------------|------------|---------------------|-----------|-------|
| Well diff. | 12 (38.7) | 14 (45.2) | 5 (16.1) | 31 |
| Moderately diff. | 47 (33.1) | 77 (54.2) | 18 (12.7) | 142 |
| Mod. poor diff. | 38 (36.9) | 57 (55.3) | 8 (7.8) | 103 |
| Poorly diff. | 13 (46.4) | 14 (50.0) | 1 (3.6) | 28 |
| Undiff. | 2 (66.7) | 1 (33.3) | 0 (0) | 3 |
| Total | 112 (36.5) | 163 (53.1) | 32 (10.4) | 307 |
| | | | $p = 0.5$ | |

3.4.5. GICA expression and flowcytometry

Figure 3 A-B shows the cumulative DI frequency and the cumulative S-phase percentage frequency in relation to GICA immunoreactivity. The graphs for negative and positive or focally positive tumours appear almost identical, indicating that neither the ploidy level nor the percentage of S-phase cells are related to GICA immunoreactivity.

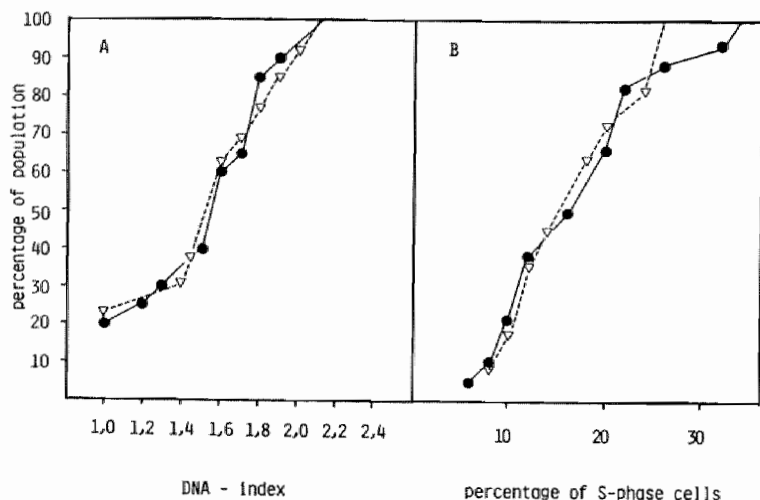


Fig. 3. DNA index (A) and percentage of S-phase cells (B) in relation to GICA expression. A. a cumulative DNA frequency is given for 13 GICA negative and 20 GICA focally positive colontumours. B. a cumulative S-phase percentage frequency is given for 11 GICA negative and 18 GICA focally positive colontumours.

3.5. Discussion

Previous studies (Koprowski et al, 1979; Atkinson et al, 1982; Arends et al, 1983) demonstrated the presence of the 1116 NS 19-9 monoclonal antibody defined monosialoganglioside in most, but not all, colorectal adenocarcinomas and therefore raised the question whether the expression of this antigen was correlated with other parameters in use for tumour staging and classification.

In a large series of colorectal carcinomas we confirmed the absence of the monosialoganglioside in about 40% of the cases, but this phenomenon did not show any statistically significant correlation with localization, stage, or degree of differentiation of the tumour nor with nuclear DNA content or percentage of cells in the S-phase of the cell cycle. In addition the distribution of GICA defined as completely positive, focally positive or negative, did not correlate with these parameters. Since 7.2% of the negative carcinomas showed a positive adjacent normal mucosa, genetically determined polymorphism as suggested by the finding of a Lewis blood group sugar sequence in the antigen (Falk et al, 1983) can not be the sole factor for absence of immunoreactivity in certain tumours.

Therefore, the phenotypic characteristics correlating with the expression of GICA in colorectal carcinomas remain obscure. Although the possibility that the antigen may be an important characteristic of neoplastic colonic epithelium cannot be entirely excluded, the results of this study indicate that the practical significance of its immunocytochemical detection for histopathological use in terms of diagnosis or prognosis is limited, because its expression is not strongly related to factors known to be correlated with prognosis, such as extension and degree of differentiation, DNA content and percentage of S-phase cells, (Dukes and Bussey, 1958; Newland et al., 1981; Chung et al., 1982; Braylan et al., 1980; Barlogie et al., 1982 and Wolley et al., 1982).

Atkinson et al. (1982) observed a loss of immunoreactivity of GICA in bone metastases of colorectal carcinomas. We observed a similar phenomenon in a large series of disseminated colorectal tumours. Whereas lymphnode metastases generally retained the antigen when present in the primary tumour, some of the studied hematogenic organ metastases appeared to have lost the antigen. This phenomenon will be subject of a next report by our group.

In due course the GICA immunoreactivity status will be correlated with the survival of the patients included in the controlled trial.

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Chapter IV

GASTROINTESTINAL CANCER ASSOCIATED ANTIGEN (GICA) IMMUNOREACTIVITY IN COLORECTAL CARCINOMA IN RELATION TO PATIENT SURVIVAL.

J.W. Arends , T. Wiggers , C. Verstijnen , J. Hilgers and F.T. Bosman .

4.1. Summary

Immunoreactivity of Gastro-Intestinal Cancer Associated antigen (GICA) was studied in tissue sections of 311 colorectal cancer patients and the results were correlated with data on patient survival. The group of uniformly GICA positive tumours (10.6%) tended to behave more aggressively than GICA focally positive (53.4%) and GICA negative tumours (36.0%), also when stratified for stage of tumour extension and histological grade. This trend, however, did not reach statistical significance, which may be the result of a bias introduced by the relatively small number of GICA positive cases.

There appeared to be no significant difference in clinical course between patients with GICA focally positive or GICA negative tumours (together comprising 89.4% of the total population studied).

Further studies including a larger number of patients and longer follow-up periods are needed to validate the suggestion that GICA positive colorectal carcinomas might show a behaviour worse than tumours displaying other modes of GICA expression. However, the results of our study suggest that GICA immunoreactivity in colorectal carcinoma tissue is unlikely to be a sensitive independent parameter for the prediction of prognosis in individual patients.

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4.2. Introduction

Gastro-Intestinal Cancer Associated antigen (GICA) is a monosialoganglioside (Magnani et al., 1981), which can be detected by a monoclonal antibody produced by the 1116NS 19-9 hybrid clone (Koprowski et al., 1979). On account of early observations GICA was considered to be of possible oncofetal nature (Koprowski et al., 1981; Magnani et al., 1981) and useful for the detection of gastrointestinal cancers (Steplewski and Koprowski, 1982). More recent studies have cast significant doubt on the oncofetal nature of the antigen (Atkinson et al., 1982; Arends et al., 1983a) and also its monosialoganglioside nature (Raux et al., 1983). Since the antigen could be detected in serum and urine of patients with colonic carcinoma (Chang et al., 1981) a RIA kit (Centocor Ca 19-9 T^M) was marketed for use in gastrointestinal cancer.

Currently, investigations are being carried out evaluating the prognostic value of serial monitoring of GICA serum levels in colorectal cancer patients. At the same time, however, it is conceivable, that the presence or absence of this antigen in colorectal carcinoma tissue may identify subgroups of tumours which show differences in behaviour in terms of patient survival.

In a previous study we have found that GICA immunoreactivity in colorectal carcinoma was not correlated with parameters known to be of prognostic significance such as localization, stage and histological grade (Arends et al., 1983b). In the present study GICA tissue immunoreactivity in tissue sections was studied directly in connection with data on patient survival.

4.3. Material and methods

4.3.1. Patients and clinical follow-up data

Tissue specimens of 311 patients entered in a multi-centre prospective controlled clinical trial (carried out from January 1979 through January 1982) aimed at comparing the value of the conventional versus no-touch surgical approach in colorectal cancer were used in this study. In addition to several clinicopathological data, follow-up data on local recurrence, occurrence of metastases and survival were consistently collected according to protocol and computerized. Survival data were corrected for non-disease-related death. Follow-up time varied from 20 to 55 months with a mean duration of 34 months. No patients were lost to follow-up.

4.3.2. Staging and grading procedures

Staging and grading were performed according to criteria outlined previously (Arends et al., 1983b).

4.3.3. Immunocytochemistry

GICA immunoreactivity was studied applying an indirect immunoperoxidase procedure on trypsinized paraffin embedded tissue sections. Details of this procedure, using 1116NS 19-9 monoclonal antibody, have been published previously (Arends et al., 1983a). Semiquantitative scoring of immunoreactivity into three categories (negative, focally positive and positive) was performed according to criteria reported before (Arends et al., 1983 b).

4.3.4. Statistical analysis

Life tables were analyzed according to the product-limit method based on individual survival times (Kaplan-Meier) and statistical calculations on the correlation of SC-immunoreactivity and survival data were done using the logrank test (Mantel/Cox) and the generalized Wilcoxon test (Breslow) with the aid of the BMDP (Biomedical Computer Program P series).

4.4. Results

4.4.1. Validity of the GICA immunoreactivity score

In order to assess the intratumour variability in GICA immunoreactivity we scored multiple immunostained sections from different parts of the tumour in 20 cases. The results (table I) show that tumours, which are uniformly GICA negative or uniformly GICA positive on one section, show very little regional variation in GICA expression. Only in one case (11*), in which the tumour consisted of foci of mucinous (GICA positive) and colloid (GICA negative) differentiation significant variability between sections occurred. In contrast, carcinomas with focal GICA immunoreactivity showed appreciable variation in the relative proportion of GICA positive cells on different sections. Therefore, focally GICA positive tumours were not further subdivided according to the relative proportion of GICA positive cells.

4.4.2. General pattern of GICA immunoreactivity

Table II shows the general pattern of GICA immunoreactivity in the population of 311 patients studied. The group of uniformly GICA positive tumours represents a minority (10.6%). The majority of the tumours showed only focal (53.4%) or no (36.0%) GICA immunoreactivity. The distribution of the cases over the various stages of tumour extension and histological grades is compiled in table III.

Table I

GICA immunoreactivity tested in multiple sections from different areas of the tumour

| Case number | negative | 0-20% | 20-40% | 40-60% | 60-80% | positive | number of blocks tested |
|-------------|----------|-------|--------|--------|--------|----------|-------------------------|
| 1 | 3 | | | | | | 3 |
| 2 | | 3 | | | | | 3 |
| 3 | | | | | | 2 | 2 |
| 4 | 8 | 1 | | | | | 9 |
| 5 | 2 | 2 | 2 | 1 | 1 | | 6 |
| 6 | | | | | | | 2 |
| 7 | 2 | 3 | | | | | 3 |
| 8 | | | | | | | 2 |
| 9 | 1 | 3 | 1 | | | 3 | 3 |
| 10 | 1 | | | | | | 5 |
| 11* | 1 | | 2 | 3 | | 1 | 2 |
| 12 | | 1 | 1 | 1 | | | 5 |
| 13 | 6 | 3 | 3 | | | | 3 |
| 14 | | | | | | | 6 |
| 15 | | | | | | | 3 |
| 16 | | | | | | | 3 |
| 17 | 5 | 5 | | | | | 5 |
| 18 | 2 | | | | | | 5 |
| 19 | | | | | | | 2 |
| 20 | | | | 1 | 1 | | 2 |

The number of sections scored according to the different categories indicated at the top of each column is given. The total number of sections studied per case is listed in the column at the extreme right hand side.

* is a mixed mucinous / colloid carcinoma.

TABLE II

GICA IMMUNOREACTIVITY IN 311 COLORECTAL CARCINOMAS

| | number/percentage | |
|-----------------------|-------------------|---------|
| GICA negative | 112 | / 36.0 |
| GICA focally positive | 166 | / 53.4 |
| GICA positive | 33 | / 10.6 |
| Total | 311 | / 100.0 |

TABLE III

STAGE AND GRADE OF 311 COLORECTAL CARCINOMAS

| Stage | number/percentage | |
|-------|-------------------|---------|
| A | 70 | / 22.5 |
| B | 119 | / 38.3 |
| C | 94 | / 30.2 |
| D | 28 | / 9.0 |
| Total | 311 | / 100.0 |

| Grade | number/percentage | |
|-----------------------|-------------------|---------|
| Well-differentiated | 31 | / 10.0 |
| Mod.-differentiated | 141 | / 45.3 |
| Poorly differentiated | 136 | / 43.7 |
| Undifferentiated | 3 | / 1.0 |
| Total | 311 | / 100.0 |

TABLE IV

P-VALUES OF THE CORRELATIONS BETWEEN THE STATUS
(POSITIVE, FOCALLY POSITIVE AND NEGATIVE)
OF GICA EXPRESSION AND SURVIVAL IN PATIENTS
STRATIFIED FOR THE VARIOUS STAGES AND HISTOLOGICAL GRADES

| | a = p (Wilcoxon) | b = p (Mantel/Cox) |
|-----------------------|------------------|--------------------|
| | a | b |
| Stage A | 0.88 | 0.74 |
| Stage B | 0.37 | 0.61 |
| Stage C | 0.19 | 0.30 |
| Stage D | 0.75 | 0.45 |
| Well-differentiated | 0.15 | 0.15 |
| Mod.-differentiated | 0.26 | 0.26 |
| Poorly differentiated | 0.33 | 0.34 |
| Undifferentiated | 0.07 | 0.06 |

4.4.3. GICA immunoreactivity in relation to patient survival

The curve relating GICA immunoreactivity to patient survival is shown in fig. 1. GICA positive patients tend to have a survival time shorter than patients with focally GICA positive and GICA negative tumours. However, this trend does not reach statistical significance (p (Wilcoxon) = 0.33, p (Mantel/Cox) = 0.25). There appears to be no difference in survival time between focally GICA positive and GICA negative patients.

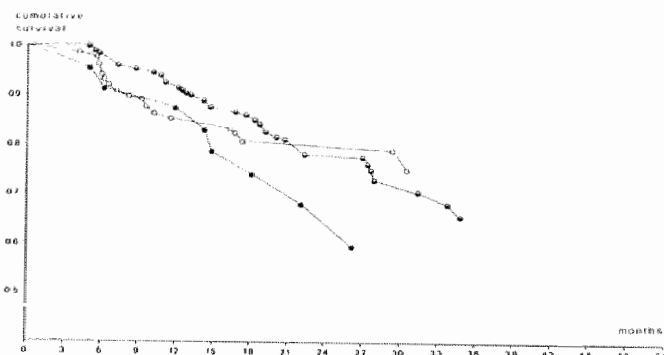


Figure 1: GICA immunoreactivity related to cumulative survival in months p (Wilcoxon) = 0.33 p (Mantel/Cox) = 0.25

○—○ GICA negative tumours. ◐—◐ GICA focally positive tumours. ●—● GICA positive tumours.

4.4.4. Stratification for stage and grade

Since the stage of extension of the tumour and the histological grade are parameters with a well established prognostic significance in colorectal cancer patients, we stratified our patient population for these parameters to determine whether a possible prognostic significance of GICA expression could have been obscured by indiscriminate testing. Even after stratification for stage and histological grade, however, GICA expression was not correlated with patient survival. Figs. 2 and 3 show the curves relating GICA expression to survival of Dukes B patients and patients with moderately differentiated tumours. The p -values for the correlations of GICA expression and survival stratified for the various stages of extension and histological grades are listed in table IV. None of these values indicate statistical significance.

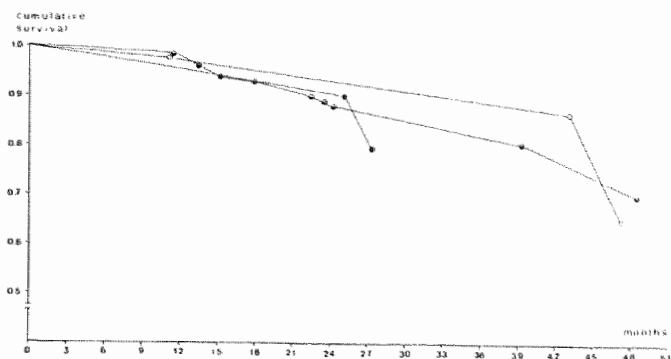


Figure 2: GICA immunoreactivity of Dukes' B patients in relation to cumulative survival. p (Wilcoxon) = 0.37 p (Mantel/Cox) = 0.61

○—○ GICA negative tumours. ●—● GICA focally positive tumours. ●—● GICA positive tumours.

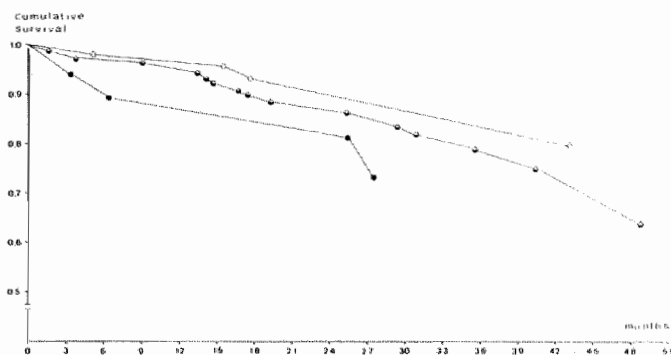


Figure 3: GICA immunoreactivity in patients with moderately differentiated colorectal carcinomas in relation to cumulative survival p (Wilcoxon) = 0.26. p (Mantel/Cox) = 0.26

○—○ GICA negative tumours. ●—● GICA focally positive tumours. ●—● GICA positive tumours.

4.5. Discussion

According to early reports the occurrence of Gastro-Intestinal Cancer Associated antigen (GICA), the 1116NS 19-9 monoclonal antibody-defined monosialoganglioside, seemed to be restricted to gastro-intestinal cancers (Koprowski et al., 1979) and fetal products (Magnani et al., 1981). Therefore it was met with high expectations as a new antigen of possible oncofetal nature, not related with CEA (Koprowski et al., 1981). Further studies, however, demonstrated GICA to occur in tumours other than of gastro-intestinal derivation and in several normal adult tissues (Atkinson et al., 1982; Arends et al., 1983a). Also, since GICA immunoreactivity in colorectal carcinoma tissue did not correlate with several parameters of well established prognostic importance, such as stage (Dukes and Bussey, 1958), histological grade (Chung et al., 1981), DNA content (Barlogie et al., 1982; Wolley et al., 1982) and percentage of S-phase cells (Braylan et al., 1980) the diagnostic and prognostic value of tissue GICA immunoreactivity seemed to be limited (Arends et al., 1983b).

The results of the present study indicate that patients with GICA positive tumours tend to have shorter survival periods than patients with tumours displaying other modes of GICA expression and therefore suggest that the presence of the GICA antigen in colorectal cancer cells might be associated with a more aggressive behaviour. Since more aggressive behaviour of tumour cells is generally associated with loss of antigen expression rather than acquisition of new antigens, GICA would be an antigen of interest in cell biological studies on the mechanisms involved in aberrant cellular behaviour. The lack of statistical significance as well as certain other methodological considerations, however, ask for cautious interpretation of our data.

In regard of these methodological considerations, firstly, it should be realized, that the median follow-up time of 34 months is rather short and may not allow identification of an appreciable difference in survival time. The influence of accepted prognostic parameters, such as stage of tumour extension and histological grade, however, usually becomes apparent within 12 months indicating that a mean follow-up duration of approximately 3 years would have to be sufficient to establish the value of GICA expression as a prognostic parameter.

Secondly, it is conceivable that the relatively small number of GICA positive tumours (accounting for only 10.6% of the total population) and the relatively small proportion of stage D or undifferentiated carcinomas biased our results.

Finally, it should be taken into account that histological evaluation of a rather limited number of sections of tumour tissue introduces a sampling error, which may also interfere with the correlations observed. For these reasons we feel, that an additional study on a larger series of GICA

positive tumours has to be conducted before final conclusions can be drawn regarding the possible relationship between tissue GICA immunoreactivity and prognosis in colorectal cancer.

That the potential prognostic value of GICA expression at the tissue level may be relevant for the clinical pathologist, however, seems unlikely. In the present series there appears to be no difference in clinical behaviour between the groups of focally GICA positive and GICA negative colorectal carcinomas and since these groups together comprise almost 90% of the total population of patients, the sensitivity of GICA expression as a single prognostic parameter apparently remains limited. Inclusion of GICA in a multivariate analysis on the prognostic significance of multiple antigens expressed in colorectal carcinomas may be rewarding. These studies are in progress.

We conclude that our initial results suggest that the presence of GICA expression in colorectal carcinoma cells is associated with a more aggressive behaviour and that as such the determination of GICA expression may be of interest in cell biological studies concerning aberrant cellular behaviour. For clinicopathological purposes the suggestion that patients with GICA positive tumours might have shorter survival times than patients with GICA focally positive and negative tumours deserves further study. In terms of sensitivity, however, the potential prognostic value of GICA expression as an independent parameter in colorectal carcinoma tissue is limited.

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Chapter V

THE VALUE OF SECRETORY COMPONENT (SC) IMMUNOREACTIVITY IN DIAGNOSIS AND PROGNOSIS OF COLORECTAL CARCINOMAS.

J.W. Arends, T. Wiggers, C.T. Thijs, C. Verstijnen, G.J.V. Swaen and F.T. Bosman.

5.1. Summary

Immunoreactivity of secretory component (SC) was tested in routinely processed tissue specimens of 314 colorectal cancer patients and correlated with clinicopathological parameters, such as stage and histological grade of the tumours as well as survival data of patients.

In 22% of the carcinomas almost all tumour cells were SC positive, 36% of the tumours showed SC immunoreactivity in a focal, heterogeneous pattern, whereas in 42% of the carcinomas SC immunoreactivity did not occur. Uniformly SC positive carcinomas demonstrated a tendency to occur more frequently in the low stage and grade carcinomas, whereas uniformly SC negative neoplasms showed the reverse trend. These trends were only statistically significant for stage. Focal, heterogeneous, SC immunoreactivity was not correlated with stage nor with grade. Furthermore, inclusion of SC heterogeneous tumours in the analysis of survival data obscured the statistical significance observed in the correlation of SC immunoreactivity status and patient survival in uniformly SC positive or negative tumours.

It is concluded that the prognostic value of SC immunoreactivity patterns in colorectal carcinoma tissue appears to be limited due to the occurrence of a substantial proportion of tumours with heterogeneous SC expression and is of less significance than staging. Nevertheless, determination of the SC immunoreactivity status may be of additional value for the identification of a subpopulation of patients with a more favourable prognosis.

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5.2. Introduction

Secretory Component (SC), a glycoprotein of 60-70 kD, is a product of most glandular epithelia (Tourville et al., 1969; Brandtzaeg, 1974; Harris and South, 1981), including the columnar cell population of the intestinal epithelium (O'Daly et al., 1971; Brown et al., 1976).

In the literature repeatedly reports have been published indicating a correlation between the presence of SC synthesis in cells in colorectal carcinoma and their histological grade (Poger et al., 1976; Weisz-Carrington et al., 1976; Rognum et al., 1980; Rognum et al., 1982; Isaacson, 1982) and stage (Rognum et al., 1980, 1982). The histological grade and stage of extension have been well established as major prognostic factors in colorectal carcinoma.

In view of the correlation of SC expression with these factors, it is conceivable that determination of SC expression in these tumours might help to predict the clinical course in individual patients. However, as yet no studies have been reported directly relating SC immunoreactivity in colorectal carcinomas to their biological behaviour, expressed in terms of patient survival.

We therefore studied SC immunoreactivity in tissue sections of a large series of colorectal carcinomas in relation to clinicopathological parameters, such as stage and histological grade of the tumours as well as clinical follow-up data of the patients.

5.3. Material and methods

The material used for the present study was derived from a multicentre prospective controlled clinical trial comprising 238 patients. This study was initiated to compare the no touch approach of colorectal carcinomas with the conventional surgical approach in terms of patient survival time and disease free interval and was conducted between January 1979 and January 1982. Sex, age, first symptoms, duration of symptoms, preoperative CEA levels, localization, stage of extension and histological grade, as well as follow-up data on local recurrence and metastases were consistently collected according to protocol and computerized.

In addition in the same period 76 patients, which could not be entered in the no-touch versus conventional surgical approach trial because of their age, the stage of extension of the tumour or the technical inability to carry out a proper no-touch procedure, were included in this study. Clinicopathological data of these patients were collected and computerized in the same way as for the trial patients. All pathological reports, histological slides and paraffin blocks were assembled in one institution (Maastricht) and reviewed with regard to stage and histological grade by two observers (J.W.A. and C.T.T.) in independent sessions. The cases in which a discrepancy occurred in the judgement of

the two observers, were reviewed jointly and a final judgement was made in mutual agreement.

5.3.1. Staging

- A - tumour confined to the bowel wall
- B - extension of tumour into pericolic fat
- C - tumour with regional lymphnode metastases
- D - tumour with distant metastases or growth into adjacent structures.

5.3.2. Grading

The histological grade of the tumours was estimated on the least differentiated areas observed in a minimum of two sections from different parts of the tumour. The applied criteria were adapted from Blenkinsopp et al. (1981) with the following modifications: a. well differentiated: tumours entirely consisting of glandular formations with up to two layers of lining cells with preserved nuclear polarity (fig. 1,a). b. poorly differentiated: tumours with more than 10% of a solid growth pattern (fig. 1,b). c. moderately differentiated: tumours covering the spectrum between a. and b. with the subdivision "moderately differentiated tending to poor" (mod. poor) i.e. carcinomas with a tendency to display solid areas (up to 10%) or a marked cribriform growth pattern (fig. 1, c,d).

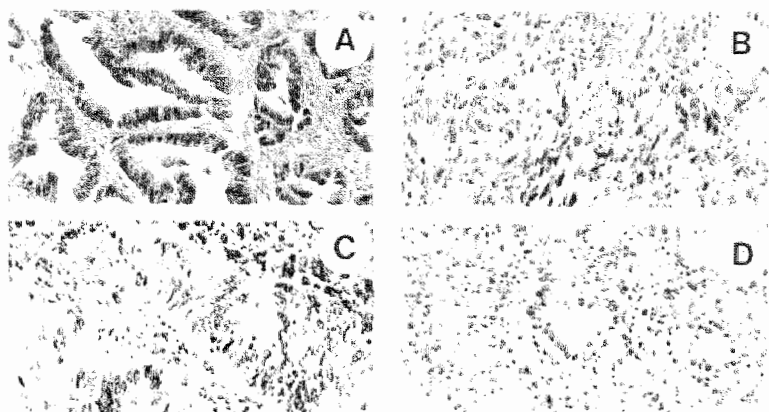


Fig. 1, a,b,c,d.

- a. Well-differentiated adenocarcinoma of colon. The tumour is made up entirely of glands with one or two layers of lining cells. H/E x 150.
- b. Poorly-differentiated adenocarcinoma of colon. The tumour largely consists of areas with a solid growth pattern. H/E x 150.
- c. Moderately differentiated adenocarcinoma of colon. The tumour shows glandular formations lined by two or more layers of atypical epithelium. H/E x 150.
- d. The tumour displays a marked cribriform growth pattern. H/E x 150.

5.3.3. Follow-up data

Survival data were corrected for non-disease-related death. Follow-up time varied from 20 to 55 months with a mean duration of 34 months. No patients were lost to follow-up.

5.3.4. Immunocytochemistry

One block of formalin fixed paraffin embedded tumour tissue, obtained from either the resection or a biopsy specimen at the time of diagnosis and preferably containing adjacent mucosa, was selected and 4 micron sections were cut. Immunocytochemical staining was performed centrally (Maastricht) in multiple staining sessions spread over 2 months, but using one single batch of antisera. Details of the staining procedure have been published (Arends et al., 1983). Briefly, the unlabeled peroxidase-antiperoxidase method was employed after prior trypsinization of the sections. The antisera used were all obtained from Dakopatts (Copenhagen, Denmark) and applied for half an hour at room temperature in the following order and dilutions: rabbit anti SC 1/1200, swine-antirabbit Ig 1/1000, rabbit peroxidase-antiperoxidase immune complexes 1/500. Between each incubation the sections were rinsed three times 5 minutes in Tris buffered saline (pH 7.6). Immunoreactive sites were visualized using diaminobenzidine.

Specificity of the anti-SC antiserum (lot nr. 031 A) was tested by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified antigen followed by immunoblotting. One band was obtained at 70 kD. Sections incubated with non-immune rabbit serum instead of anti SC immune serum served as negative control. Adjacent colonic mucosa served as an internal positive control reference.

5.3.5. Scoring of immunoreactivity

Scoring of immunoreactivity was performed using the following definitions:

- tumours displaying immunoreactivity in over 80% of all tumour cells - positive (fig. 2, a).
- tumours demonstrating immunoreactivity in 5-80% of the tumour cells - focally positive (fig. 2, b).
- tumours reactive in less than 5% of the tumour cells - negative (fig. 2, c).

In positive and focally positive tumours no distinction was made in the mode of SC expression (i.e. staining of the apical plasma membrane or diffusely cytoplasmic).

Histological grading and scoring of immunoreactivity of the tumour were done in independent sessions.

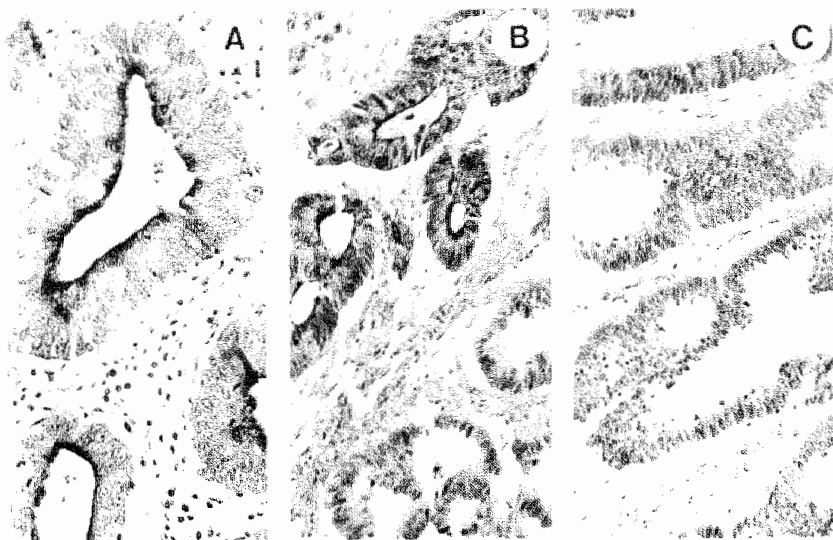


Fig. 2, a,b,c

a. SC-positive colonic adenocarcinoma with expression of the antigen at the luminal plasmamembrane and diffusely over the cytoplasm of the epithelium. Immunoperoxidase x 200. b. SC-focally positive colonic adenocarcinoma. The glandular formations in the upper part of the picture express SC where as the antigen is absent in the apical membrane and cytoplasm of the neoplastic elements at the bottom. Immunoperoxidase x 150. c. SC-negative colonic adenocarcinoma without detectable immunoreactivity for the antigen. Immunoperoxidase x 150.

5.3.6. Statistical analysis

Differences observed in the cross tabulations between SC immunoreactivity and stage as well as grade were tested on their significance with a raw chi-square test for association and p-values were calculated with the aid of SPSS (Statistical Package for Social Sciences).

The life tables were analysed according to the product limit method based on individual survival times (Kaplan-Meier). Calculation of the p-values for statistical evaluation of the differences in regard of the pattern of SC-immunoreactivity was performed with the logrank test (Mantel-Cox) and the generalized Wilcoxon test (Breslow) using the BMDP (Bio-medical Computer Program P-series).

5.4. Results

5.4.1. Validity of staging and grading procedures

In order to establish to what extent our staging and especially our grading definitions enabled us to distinguish groups of patients differing in prognosis we first correlated these parameters with the clinical follow-up data. In figures 3 and 4 the curves relating stage and grade of tumours to survival of patients are shown. These figures show that patients with stage C and stage D tumours, together comprising 39% of the total population demonstrate significantly shorter survival than patients with stage A and B tumours (p (Wilcoxon) 0.0001, p (Mantel/Cox) 0.0001). Also, poorly differentiated and undifferentiated tumours, together accounting for 10% of the total population, are associated with shorter survival times in comparison with well- and moderately differentiated tumours (p (Wilcoxon) 0.0001, p (Mantel/Cox) 0.0001).

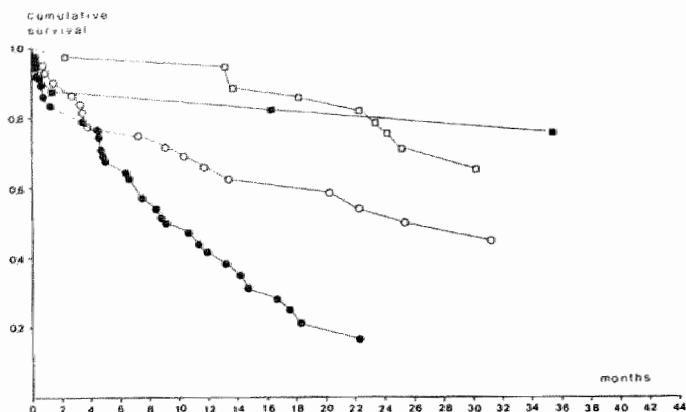


Fig. 3

Cumulative survival in relation to stage of tumour extension.

■—■ stage A, □—□ stage B,
 ○—○ stage C, ●—● stage D.

Stage C and D tumours (39% of the population) are seen to have survival times different from the rest of the tumours (p (Wilcoxon) 0.0001, p (Mantel/Cox) 0.0001).

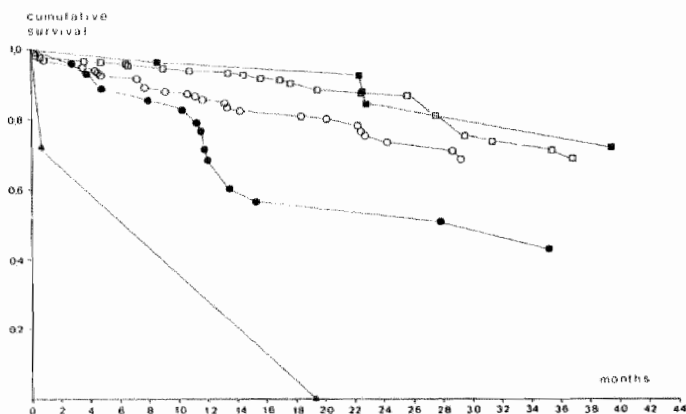


Fig. 4

Cumulative survival in relation to histological grade: ■—■ well differentiated, □—□ moderately differentiated,
 ○—○ moderately differentiated tending to poor,
 ▲—▲ poorly differentiated, undifferentiated.

Undifferentiated and poorly differentiated carcinomas show a clinical course distinct from the rest of the tumours (p (Wilcoxon) 0.0001, p (Mantel/Cox) 0.0001).

5.4.2. General distribution of SC immunoreactivity

Table I shows the general distribution of SC immunoreactivity in the 314 cases included in this study. The tumours demonstrated either a uniformly positive (22%) or a focally positive (36%) staining pattern, whereas 42% showed uniform absence of SC.

TABLE I
SC-IMMUNOREACTIVITY IN 314 COLORECTAL CARCINOMAS

| | number | percentage |
|------------------|--------|------------|
| Negative | 132 | 42 |
| Focally positive | 113 | 36 |
| Positive | 69 | 22 |

5.4.3. SC immunoreactivity and stage/grade

In tables II and III the data on SC expression in relation to stage and histological grade are compiled. SC negative tumours occurred more often in stages C and D (50-55%) and in the higher histological grades (47-64%) than in the group as a whole (42%). Conversely, SC positive tumours occurred more frequently in stages A and B (32-25%) and in histologically low-graded tumours (29-28%) compared to the whole group (22%). These findings reached statistical significance in respect of stage ($p = 0.017$), but not for grade ($p = 0.09$).

Focal SC immunoreactivity was not correlated with the stage nor with the grade of colorectal carcinomas.

Table II

SC-immunoreactivity and stage

| | negative number/percentage | focally positive number/percentage | positive number/percentage | total number/percentage |
|---|-------------------------------|---------------------------------------|-------------------------------|----------------------------|
| A | 27 / 38 | 21 / 30 | 23 / 32 | 71 / 23 |
| B | 42 / 35 | 48 / 40 | 30 / 25 | 120 / 38 |
| C | 47 / 50 | 34 / 36 | 13 / 14 | 94 / 30 |
| D | 16 / 55 | 10 / 35 | 3 / 10 | 29 / 9 |
| | | | | 314 |

*p (raw chi-square = 0.017)

Table III

SC-immunoreactivity and grade

| | negative number/percentage | focally positive number/percentage | positive number/percentage | total number/percentage |
|-----------------|-------------------------------|---------------------------------------|-------------------------------|----------------------------|
| wel. diff. | 10 / 32 | 12 / 39 | 9 / 29 | 31 / 10 |
| mod. diff. | 50 / 35 | 53 / 37 | 39 / 28 | 142 / 46 |
| mod. poor diff. | 49 / 47 | 38 / 36 | 18 / 17 | 105 / 34 |
| poorly diff. | 18 / 64 | 8 / 29 | 2 / 7 | 28 / 9 |
| undiff. | 2 / 64 | 1 / 33 | 0 / 0 | 3 / 1 |
| | | | | 309 |

p (raw chi-square = 0.09)

5.4.4. SC immunoreactivity and patient survival

The group of patients with uniformly SC positive tumours demonstrated a significantly better survival than the groups of patients with focally SC positive and SC negative tumours taken together (p (Wilcoxon) 0.02, p (Mantel/Cox) 0.02). Also, when SC positive tumours were compared with SC negative tumours alone a significant difference in survival was found in favour of patients with SC positive tumours (p Wilcoxon) 0.017, p (Mantel/ Cox) 0.022) (fig. 5).

However, comparison of the group of uniformly SC positive and focally SC positive tumours with uniformly SC negative tumours did not reveal a significant difference in patient survival (p (Wilcoxon) 0.16, p (Mantel/Cox) 0.21). No significant difference between uniformly SC negative and focally SC positive tumours could be noticed (p (Wilcoxon) 0.80), p (Mantel/Cox) 0.89).

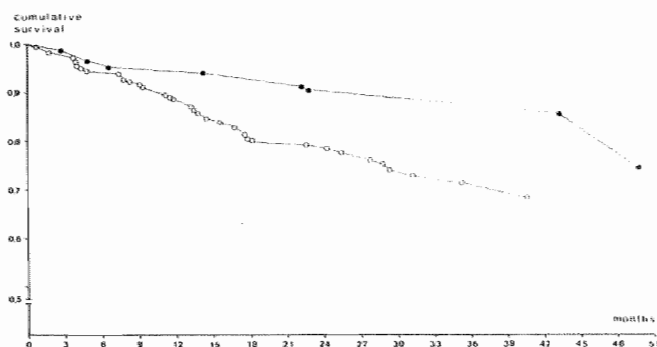


Fig. 5
Cumulative survival in relation to SC immunoreactivity state ●—●
SC positive, ○—○ SC negative p (Wilcoxon) = 0.017, P (Mantel/Cox) =
0.022. SC focally positive carcinomas are excluded in this analysis.

5.5. Discussion

Previous immunocytochemical studies on the expression of SC in colorectal carcinomas have shown that well-differentiated carcinomas tend to contain more SC positive cells than poorly-differentiated variants. Also a tendency for SC positive tumours to occur more frequently in the early Dukes' stages has been noticed. Since the extension of the tumour (Dukes and Bussey, 1958; Newland et al., 1981) and -to a lesser extent - histological grade (Newland et al., 1981; Chung et al., 1982) are known to be important factors for prognosis in colorectal carcinoma, the results of the studies reported so far in the literature warrant the supposition that determination of the status of SC expression may be of additional value for prediction of the clinical course in individual patients.

The aim of the present study was to reconfirm the correlation of SC expression with stage and histological grade on a large series of colorectal carcinoma patients. In addition we intended to correlate tissue SC immunoreactivity with clinical follow-up data of the patients.

The results of this study largely confirm and extend earlier findings. SC uniformly positive carcinomas demonstrated a tendency to occur more frequently in the A and B stage and low graded carcinomas, whereas uniformly SC negative neoplasms showed the reverse trend, which however only appeared to reach statistical significance in respect of tumour stage. In contrast, focally SC positive carcinomas did not show any correlation with stage nor with grade. Patients with uniformly SC positive tumours demonstrated significantly longer survival than patients with SC negative tumours. Inclusion of focally SC positive tumours in the analysis of survival data, however, obscured this statistical significance.

In terms of specificity stage of extension and histological grade are better prognostic indicators than SC immunostaining: the differences in prognosis between stage A and D tumours or between well differentiated and poorly differentiated tumours is much more marked ($p < 0.0001$) than that between uniformly SC positive and SC negative tumours ($p < 0.017$). In terms of sensitivity SC immunostaining as prognostic indicator appears to take an intermediate position between stage of extension and grade: staging identified 39% of the patient population as prognostically different, SC immunostaining identified 22% and grading only 10%. We feel therefore, that the SC immunoreactivity status is a better prognostic variable than the histological grade, but also that its prognostic value is inferior to that of the stage of extension of the tumour.

In comparing our findings with those reported by Poger et al. (1976), Rognum et al. (1980, 1982) and Isaacson (1982) on the same subject, it should be realized, that there are certain differences in the applied techniques. Firstly, it

should be taken into account that we used an immunoperoxidase procedure on formalin fixed and routinely processed tissue to detect SC immunoreactivity, whereas Poger et al. and Rognum et al. applied an immunofluorescence procedure on mildly acetone fixed frozen material. However, we do not believe that this difference in technique is of major importance, since the pattern of SC immunoreactivity we observed is identical to that reported by these authors. Furthermore, the findings of Isaacson who used a procedure similar to ours, are not in contradiction with those of other workers. Another aspect, in which our study differs from others, is the system of scoring of immunoreactivity. Whereas Rognum et al. and Isaacson scored not only the cell numbers but also the intensity of the immunoreaction as a measure of SC expression, we felt that on our material a similar approach was unjustifiable since routine fixation and embedding procedures may variably affect the immunoreactivity of tissue bound SC, especially in surveys including tissue specimens processed in different centers and sections immunostained in multiple staining sessions. We therefore restricted ourselves to scoring of the number of immunoreactive cells as a measure of SC expression in a tumour.

An important aspect to be considered in the search for prognostic indicators at the histological level is the phenomenon of phenotypic heterogeneity of tumour cells, which in colorectal carcinomas is known to occur in respect of antigen expression (Prehn, 1970; MacLean et al., 1982; Arends et al., 1984) and histological grade (Qualheim and Gall, 1953). For practical purposes a simple overall classification of SC expression and histological grade is usually performed. Such an overall approach, however, obscures potentially relevant differences between individual tumour cells. Overall classification of a well differentiated tumour as prognostically unfavourable in relation with absence of SC immunoreactivity exemplifies this problem. SC producing columnar cells represent only one of the functionally differentiated cell types in colonic mucosa. Other differentiated cells include goblet cells and enteroendocrine cells. It may be hypothesized that only part of the well differentiated tumours are composed of columnar cell-like SC producing cells and that there are other well differentiated tumours with a relatively benign behaviour which are predominantly composed of goblet-like cells (e.g. mucinous, signet ring cell and colloid carcinomas) or enteroendocrine cells (e.g. carcinoids and neuroendocrine carcinomas).

In summary, our data demonstrate that patients with uniformly SC positive tumours (representing about one fifth of the total patient population) show a better survival than patients with focally SC positive or SC negative tumours. Therefore determination of the SC immunoreactivity status in colorectal tumours may be of value for the identification of a subpopulation of patients with a more favourable prognosis.

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Chapter VI

TUMOUR CELL HETEROGENEITY IN PRIMARY AND METASTATIC COLO-RECTAL CARCINOMA.

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G.J.V. Swaen , J. Hilgers , E. Lennox and Z. Steplewski

6.1. Summary

In order a) to assess the extent of phenotypic heterogeneity in terms of antigenic composition of primary colorectal carcinomas, b) to detect possible differences in antigenic composition between primary tumours and their metastases and c) to study the suitability of the use of antibodies as targeting-vehicles for the purpose of radioimmunodiagnosis and therapy, we studied a series of disseminated colorectal carcinomas for the presence of various colorectal (cancer) tissue associated-antigens, using a panel of conventional and monoclonal antibodies in an immunoperoxidase technique. Primary tumours expressed a multitude of antigens with marked intercellular heterogeneity; their metastases appeared to be no less heterogeneous in this respect. In general regional lymphnode metastases appeared to express the same antigens as their primary tumours, whereas haematogenous metastases tended to loose expression of some antigens. Since many antigens were found to be only focally expressed it may be necessary to use combinations of targeting-antibodies for effective visualization or toxification of the antigenically diverse tumour cell subpopulations in a given tumour. Immunoperoxidase studies are mandatory for the documentation of the cellular distribution of the antigens to be selected as targets for radioimmunodiagnostic and therapeutic procedures.

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6.2. Introduction

In recent years much attention has been focussed on the development of techniques to detect and localize malignant neoplasms by means of radiolabeled antibodies in combination with scintigraphic scanning methods (Mach et al., 1974, Primus and Goldenberg, 1980). It has become apparent, that the efficacy of these radioimmunodiagnostic procedures depends on several factors, such as the specificity (Searle et al., 1981), affinity (Begent et al., 1980) and Ig class of the targeting-antibody, the accessibility of the target-antigen (Moshakis et al., 1981), the amount of free target-antigen in serum and interstitial fluid, which potentially blocks tissue binding of the radio-labeled antibody (Primus and Goldenberg, 1980), and finally the choice of the isotope and the labeling procedure (Epenetos et al., 1982). In addition, a potential problem, to which hitherto very little attention has been paid, may be formed by the intercellular phenotypic heterogeneity in malignant neoplasms. Evidence has accumulated, that, by the time they can be clinically diagnosed, human neoplasms consist of subpopulations of cells with heterogeneous phenotypic features, including DNA-content (Vindelov et al., 1980) growth rate (Butel et al., 1977), antigenic composition (Prehn, 1970) and response to cytotoxic drugs (Barranco et al., 1973), regardless of their generally assumed monoclonal origin. Also there are indications that primary tumours consist of subclones of cells differing in metastatic potential (Fidler and Kripke, 1977). It is not unlikely that in the evolution of metastatic tumours clonal selection takes place and therefore it is conceivable that the antigenic composition of neoplastic cells in metastases is more restricted than in the parental tumours. Such a phenomenon would be relevant to study with regard to the efficacy of application of radioimmunodiagnostic - and especially immunotherapeutic procedures, since these rely on the interaction of the antibody with as many tumour cells as possible. Therefore, the tissue distribution of the target-antigen of antibodies, considered for targeting purposes, has to be documented carefully, not only in respect of the possible presence of its determinant(s) in normal tissue components, but also in connection with the extent of its expression in primary tumours and their metastases. So far most studies on tissue binding of targeting antibodies were performed on human cancer cells in tissue culture or xenografted in nude mice. Under these artificial conditions human tumours may not retain the original pattern of expression of cellular antigens (Rutzky and Siciliano, 1982) and therefore immunocytochemical surveys of tumour tissue specimens are essential to fully document the pattern of antigen expression in the *in vivo* situation.

We therefore studied the expression of various colorectal-(cancer)tissue-associated antigens in a large series of primary colorectal carcinomas as well as in their regional lymphnode metastases and a few hematogenous metastases using

a variety of conventional and monoclonal antibodies in an immunoperoxidase technique. Special attention was paid to: a) the extent of intercellular antigenic heterogeneity of primary colorectal tumours, b) the possible differences in antigenic composition between primary and metastatic tumours and c) finally the suitability of the applied antibodies as targeting vehicles for radioimmunodiagnostic and therapeutic purposes.

6.3. Materials and methods

6.3.1. Material

All tumour tissues were derived from patients with metastatic colorectal carcinoma collected in a multicentre prospective controlled trial, conducted from january 1979 till january 1982 and comparing the value of standard surgical procedures with a no-touch approach.

87 Cases with regional lymphnode metastases and 15 cases with hematogenous metastases were collected.

All tissue specimens were fixed in 4% neutral buffered formalin, embedded in paraffin and serially sectioned at 5 micron.

6.3.2. Nature of the antibodies

Conventional rabbit anti CEA and rabbit anti secretory component (SC) antibodies were purchased from Dako-immunoglobulins (Copenhagen, Denmark).

Designation, immunogen, target-antigen, and source of the used monoclonal antibodies are compiled in Table I.

Table I

Colorectal (cancer) tissue reactive monoclonal antibodies

| Designation | Immunogen | Target antigen | Source |
|---------------------------------------|--|-------------------------------|-------------------------------|
| 1. a. PARLAM I | a and b, plasma membrane | epitope on CEA (like)molecule | Verstijnen et al. (1983) |
| b. YPC ₂ /12.1 | preparations of colonic carcinomas | | Finan et al. (1982) |
| c. CE ₆ /2D _{3.1} | highly purified CEA | | Alderson (unpublished) |
| 2. YPC ₂ /44.3 | plasma membrane preparations of colonic adenocarcinoma | mucin - like | Finan et al. (1982) |
| 3. 1116 NS ₁₉₋₉ | SW 1116 colonic carcinoma cell line | monosialoganglioside | Koprowski et al. (1979) |
| 4. WGHS ₂₂₋₂ | freshly isolated gastric carcinoma cells | unknown | Steplewski & Koprowski (1982) |
| 5. 67 D ₁₁ | human milk fat membranes | of glycolipid nature | Hilkens et al. (1982) |
| 6. MH ₂ /6D ₄ | HT-29 colon carcinoma cell line | blood group A | Voak et al. (1980) |

6.3.3. Immunocytochemistry

In order to evaluate the effect of routine tissue processing procedures on the preservation of the target-antigens the antibodies were initially tested on frozen sections of colorectal tumours, postfixed in acetone (10 min, 4°C) and the staining result was compared with that of trypsinized paraffin sections of the same tumours. With the exception of YPC₂/44.3 all antibodies showed comparable patterns of immunoreactivity with these two procedures.

The target-antigen of YPC₂/44.3 could be demonstrated in non-trypsinized paraffin sections in a distribution analogous to that of frozen sections. The conventional antibodies were applied in the unlabeled peroxidase-antiperoxidase procedure, whereas the monoclonal antibodies were employed in an indirect peroxidase-labeled antibody procedure, using rabbit-antimouse Ig (Dako, Code nr. Pl69) as the second layer. The details of these procedures have been reported elsewhere (Arends et al., 1983 a + b).

6.3.4. Scoring of immunoreactivity

Immunoreactivity of all antibodies was semiquantitatively scored in primary tumours and metastases as positive (over 80% of the tumour cells expressing the antigen), negative, (less than 5% of tumour cells expressing the antigen) or focally positive (tumours showing reactivity in between 5 and 80% of the neoplastic cells).

6.4. Results

6.4.1. General characteristics of antigen localization and mode of expression

In the present study the antibody binding patterns were comparable in the primary tumours and in their metastases. Differences were noticed only with regard to their extent of expression. Conventional anti-CEA antibodies and the three monoclonal anti-CEA antibodies showed analogous immunoreactivity in primary tumours as well as in metastases, their reaction products being localized in luminal cell debris and the apical plasma membrane, or diffusely in the cytoplasm but with increased concentration in the apex. The majority of the primary tumours and metastases showed CEA immunoreactivity in most cells (93.2% positive, 6.4% focally positive, 1.3% negative).

Anti-SC and the used monoclonal antibodies showed immunoreactivity diffusely in the cytoplasm, in the apical cytoplasm or on the plasma membrane. Antigen expression was significantly more often focal than was found for CEA (36.1 - 53.1% as against 6.4% for CEA)

6.4.2. Characteristics of antigen expression in primary tumours and regional lymphnode metastases

The difference in antigen expression between 87 primary

colorectal carcinomas and their regional lymphnode metastases are given in table II.

Table II

Differences in antigen expression of primary colorectal carcinomas and regional lymphnode metastases

| Number of cases | Number of antigens differing in expression pattern | Percentage of cases differing in expression pattern |
|-----------------|--|---|
| 34 | - | 39.0 |
| 28 | 1 | 32.3 |
| 15 | 2 | 17.2 |
| 8 | 3 | 9.2 |
| 2 | 4 | 2.3 |

71.3

It is clear that in the majority of cases (71.3%) no differences in antigen expression or a difference for only one antigen occurred. Qualitative differences (positive to negative or vice versa) accounted for only 10% of the changes. In all other cases the change was from positive to focally positive, focally positive to negative or vice versa (table III).

Table III

Pattern of alteration in antigen expression in primary colorectal carcinoma and regional lymphnode metastases

| Change in antigen expression | Number of observations |
|------------------------------|------------------------|
| positive / negative | 9 |
| positive / focally positive | 37 |
| focally positive / negative | 44 |

If changes from focally positive to positive and from negative to focally positive are considered to indicate increased antigen expression, 15 primary tumours expressed antigens more extensively than their lymphnode metastases, whereas in 23 cases the lymphnode metastases expressed antigens more extensively than their parental tumours. The remaining 15 cases showed both phenomena simultaneously for different antigens. Concordance and discordance for the individual antigens in primary tumours and their lymphnode metastases are summarized in table IV.

Table IV

Concordance of antigen expression in primary colorectal carcinomas
and regional lymphnode metastases

| | percentage of concordance | number of differing observations | expression in primary more than metastases | expression in metastases more than primary |
|-------------------------|---------------------------------|--|---|---|
| SC | 78.2 | 19 | 9 | 10 |
| CEA | 98.8 | 1 | 1 | 0 |
| 1116 NS ₁₉₋₉ | 73.6 | 23 | 8 | 15 |
| WGHS ₂₂₋₂ | 78.2 | 19 | 10 | 9 |
| YPC ₂ /44.3 | 78.2 | 19 | 11 | 8 |
| 67 D ₁₁ | 87.4 | 11 | 5 | 6 |

It is clear, that concordance for the individual antibodies was the predominant pattern and that, where differences were observed, primary tumours showed more extensive as well as more limited patterns of expression compared with their metastases.

6.4.3. Characteristics of antigen expression in primary tumours and distant and/or hematogenous metastases
The number of available hematogenous metastases that could be studied was small (table V).

Table V

Antigen differences noted between primary colorectal carcinomas
and organ metastases

| | site of metastases | antigen difference noted - no difference noted |
|---|-----------------------|--|
| Dukes D patients | 1. liver | YPC ₂ /44.3 from focal to negative |
| | 2. liver | SC and WGHS ₂₂ from focal to negative |
| | 3. hilus of spleen | - |
| metastases developed during follow-up | 4. omentum | - |
| | 5. praesacral region | WGHS ₂₂₋₂ from negative to focal |
| | 6. right adnex | - |
| | 7. skin | - |
| | 8. liver | WGHS ₂₂₋₂ and 1116 NS ₁₉₋₉ from focal to negative |
| autopsy cases | 9. liver | SC from focally positive to negative |
| | 10. liver | idem |
| | 11. liver + uterus | idem |
| | 12. liver + lung | idem |
| | 13. brain | idem |
| | 14. lung | - |
| | 15. ovary | - |

It is clear, that where differences in antigen expression were noticed organ metastases tended to display less antigens. This phenomenon was most strikingly observed in liver metastases, especially in regard of SC.

6.5. Discussion

Two technical limitations may be relevant to the validity of the results of the present study. The first concerns the possibility of sampling error. For practical reasons only one block of the primary tumour and of the metastases was selected for immunocytochemical staining. Conceivably studying multiple blocks might result in even more extensive variability in the expression of antigens in primary tumours. However, of a limited number of primary tumours an extensive survey was performed on multiple sections and comparing these results with those obtained on one representative block only did not reveal significant differences.

Of the metastatic localizations also only one block was studied, which often contained only a limited amount of tumour tissue. Another potential source of sampling error is the fact that only a limited number of hematogenous metastases was available. Our impression, that hematogenous metastases tend to display a more limited pattern of antigen expression than their primary tumours therefore needs to be reconfirmed in a much larger series.

A second technical limitation is related to the fact that most of the available hematogenous metastases were obtained at autopsy. Due to autolysis immunohistochemical profiles of autopsy material might not completely reflect the in vivo situation. Based on our experience with the immunoreactivity of intestinal antigens in autopsy specimens of intestinal mucosa in comparison with surgical specimens (unpublished observations), however, it is our impression that autolysis is not a significant factor in the reduction of antigen expression, which we observed in hematogenous metastases.

We therefore feel that, even if these limitations are taken into consideration, the conclusion that primary colorectal carcinomas as well as their regional lymphnode metastases display marked intercellular phenotypic heterogeneity is warranted. At least two mechanisms may be involved in the generation of phenotypic heterogeneity in metastatic lesions. Firstly it is conceivable that metastases do not originate from emboli of single tumour cells but rather from clumps of tumour cells with varying phenotype. Secondly, it is not unlikely that metastasising tumour cells are relatively undifferentiated and may possess pluriform differentiating potential (Fidler and Hart, 1982). Whether one of these two mechanisms or even a combination of both is responsible for the observed phenotypic heterogeneity in metastases remains to be elucidated.

Whether distant metastases really tend to loose expression of differentiation antigens, such as SC, remains to be established in a larger series of cases. Though this phenomenon would be difficult to explain in terms of increasing heterogeneity of metastases during the course of their development, selective loss of immunoreactivity has been reported (Atkinson et al., 1982), however, not especially in the liver, where metastases in our study showed a remarkable loss of the antigens studied.

The finding of an over-all conservation of antigen expression in lymphnode metastases, and for most antigens also in distant metastases, confirms that in principle immunotargeting procedures might be of use for diagnosis and treatment of human tumours. The heterogeneity in expression of different antigen however, deserves ample consideration. Most of the antibodies employed show a focal distribution pattern of immunoreactivity in the primary tumour as well as their metastases, implying that immunotargets as a rule are not to be expected to be expressed on all cells of the malignant neoplasm. Of the target-antigens studied, CEA appeared to be most constantly expressed by the cells of both primary and metastatic tumours, though its distribution in normal intestinal mucosa and its release into body fluids are disadvantages for use in immunotargeting procedures.

For reliable visualization or effective toxification of the antigenically diverse tumour cell populations in a given tumour it will probably be necessary to use combinations of immunotargeting vehicles so chosen that the total of its respective target-antigens cover most of the areas of tumour growth. In the documentation of the distribution of these target-antigens and the subsequent selection of antibodies appropriate for use in radioimmunodiagnostic and therapeutic procedures immunocytochemical surveys are mandatory.

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Chapter VII

EPILOGUE

7.1. Introduction

The identification and localization of antigens in cancerous tissues entails apparent perspectives, but also is subject to certain limitations. In this epilogue both limitations and perspectives of antigen expression at the tissue level will be discussed in the context of the data obtained in our investigations. At the end of the chapter possible ways to alleviate the limitations of antigen expression studies are suggested and finally the perspectives of study of antigen display in respect of a cell-origin-related classification are indicated.

7.2. Summary of the results of the study

In Chapter I the potential value of tissue antigen expression for the classification and characterization of primary tumours and metastatic lesions as well as for the assessment of grade of malignancy of colorectal neoplasms is outlined. Also, the problem of antigen-specificity and the phenomenon of intercellular phenotypic heterogeneity of antigen display are discussed as possible impediments in the establishment of significant associations between antigen expression and tumour type or biological behaviour.

The studies presented in this thesis emphasize the interrelation of these important aspects in the study of antigen expression, especially in connection with secretory component (SC) and the monoclonal antibody 1116 NS 19-9 defined monosialoganglioside (GICA) in colorectal carcinomas.

In Chapter II the potential value of GICA for the diagnosis of colorectal carcinomas is addressed because it initially was considered a colorectal carcinoma-specific antigen. By immunoperoxidase studies on mildly fixed frozen tissue sections and routinely fixed paraffin-embedded sections the antigen was shown to be present in many glandular epithelial cells and variety of tumours of other than gastrointestinal tract origin. The results of the study indicate that GICA cannot be considered a colorectal carcinoma-specific antigen. Consequently, its value as a diagnostic tool in these tumours is very limited.

Chapters III, IV and V deal with the search for associations of GICA and SC expression with clinicopathological variables known to be of prognostic significance in colorectal carcinomas, such as stage of tumour extension and histological grade. In addition the immunoreactivity status of both antigens was directly correlated with data on patient survival. The mode of GICA expression did not appear to be correlated with stage or grade of colorectal carcinomas (chapter III), nor with other parameters studied such as lo-

calization, DNA content and percentage of S-phase cells. The antigen demonstrated a focal, heterogeneous expression pattern in about half of the carcinomas and was present on most tumour cells in only about 10% of the investigated tumours. Patients with uniformly GICA positive tumours tended to show shorter survival periods than patients with tumours displaying other types of GICA expression. This trend, however, did not reach statistical significance (Chapter IV). Although this finding suggests a possible association between the presence of GICA and a more aggressive behaviour, it should be interpreted with caution because of the small number of GICA uniformly positive cases in the series, the relatively short follow-up periods and certain methodological limitations. One such limitation was formed by the circumstance that for immunohistochemical studies in the majority of cases only one block of tumour tissue was available. In a study on multiple sections of a limited number of carcinomas scoring of uniformly positive and negative tumours appeared to be rather reliable on account of one section. Carcinomas displaying GICA in a focal, heterogeneous pattern, however, appeared to show a wide variation in the number of immunoreactive cells in various areas of the tumour. Therefore exact scoring of the percentage of positive cells in heterogeneous tumours in one section appeared to be unjustifiable to acquire a correct impression on the number of immunoreactive cells in the tumour as a whole. Thus heterogeneity in GICA expression introduced a source of sampling error in our experimental design which prevented us from the execution of further studies on the survival periods of patients with tumours in which a more accurate immunoreactivity scoring was performed. That the phenomenon of intercellular phenotypic heterogeneity interferes with studies attempting to establish associations of antigen expression with other clinicopathological variables is even more explicitly exemplified by our data on the immunoreactivity of SC in colorectal tumours (Chapter V). Carcinomas with uniformly positive SC expression frequently occurred in the groups of low-stage and low-grade carcinomas, whereas the reverse trend was observed in SC negative tumours. The substantial proportion (36%) of SC heterogeneous tumours, however, failed to show any correlation with stage and grade. In addition, inclusion of the group of SC heterogeneous carcinomas in the analysis of patient survival data obscured the significant difference in survival observed between patients with SC uniformly positive and negative tumours. Thus, although our data establish SC expression as an independent parameter of prognostic relevance in colorectal carcinoma, the sensitivity of the parameter was reduced due to the occurrence of a group of tumours with heterogeneous SC expression.

In Chapter VI a study on the extent of heterogeneity in antigen expression of primary and metastatic colorectal carcinomas using a panel of colorectal carcinoma reactive antibodies is reported and at the same time the hypothesis,

that clonal selection during the process of metastasis might be reflected in differences in antigen display between parental and metastatic tumours was tested in connection with a possible interference of such a phenomenon with immunotargeting procedures. The antigenic composition of primary colorectal carcinomas as well as their metastases appeared to be diverse. Lymphnode metastases generally retained the antigenic profile observed in the parental tumours, whereas haematogenous metastases tended to loose expression of some antigens. A definite reflection of clonal selection in metastasis on antigen display, however, was not obtained for the studied antigens. The extensive heterogeneity in expression of most antigens necessitates the use of combinations of targeting-antibodies for effective visualization or toxification of tumour cells in immunotargeting procedures.

In summary, the important messages of our studies are, that

- a) immunocytochemical characterization of antigen expression on a variety of different tissues is an essential step in the exploration of antigen-specificity and the selection of target-antigens suitable for immunotargeting procedures.
- b) determination of tissue expression of certain antigens can be of prognostic value, although the occurrence of heterogeneity in antigen display may attenuate the sensitivity of such prognostic significance.

7.3. Possible alleviating approaches to limiting aspects

7.3.1. Antigen-specificity

Concerning the limitations posed by antigen-specificity two main aspects deserve consideration: the apparent lack of colorectal carcinoma-specific antigens and the procedures involved in the determination of antigen-specificity.

In spite of five years of intensive research with monoclonal antibodies the existence of tumour-specific antigens in human colorectal carcinomas is still unproven. This may be partly due to the relatively aselective immunization procedures so far used in the generation of monoclonal antibodies. An approach, in which some form of prior antigen selection has been applied might be more rewarding. Selection of membrane glycoproteins of colorectal cancer cells through lectin affinity or lipid extraction followed by further selection through thin-layer chromatography, for example, might increase the chance of obtaining tumour-specific antibodies. Alternatively, the immune response in recipient animals could conceivably be more restricted to tumour-specific antigens, when these animals could be rendered tolerant to normal colonic mucosal antigens by repeated low-dose administration with extracts of normal colonic mucosa followed by immunization with extracts of colonic cancer tissue.

In most biomedical research institutes antigen-specificity is often determined on cancer cells in tissue culture. Although this exploration is of apparent value as a first screening on antigen-specificity it should be complemented with similar studies on a wide variety of fresh or fixed tissues. Cultured cancer cells probably represent only a limited selection of the cells present in the original tumours and may in addition lose some characteristics of the original tumour during cultivation, whereas fresh tissue can be expected to express the almost full range of antigens present in the *in vivo* situation. Therefore close cooperation between basic scientists and surgical pathologists would greatly enhance and facilitate appropriate antigen-specificity testing.

7.3.2. Phenotypic heterogeneity

That phenotypic heterogeneity of antigen display forms a major obstacle in studies aimed at establishing associations between antigen expression and other clinicopathological variables has been shown in the literature and explicitly in our investigations. Especially studies relying on the comparison of two variables with intrinsic heterogeneity are liable to be flawed by a simple categorization of either of these variables. This situation occurs in the correlation of antigen expression with histological grade (Qualheim and Gall, 1953), where simple over-all classification of tumours into one grade and one type of antigen expression may obscure a potential correlation between the two variables at the level of the individual cell.

There are two possible ways to diminish the interference of antigenic heterogeneity in the establishment of associations between antigen expression and other prognostic parameters.

Firstly, since the survival period of individual patients can be accurately determined, correlation of antigen expression with survival in patients only leaves the variable nature of the parameter of antigen display. This type of potentially rewarding studies, however, hitherto has been scarcely conducted due to the reliance on a large series of clinically carefully documented patients, requiring a close multidisciplinary approach of clinicians and pathologists.

Secondly, the attenuating effect of antigenic heterogeneity on the sensitivity of antigens with value as single prognostic indicators conceivably could be largely compensated for by application of a number of such antigens in a multivariant analysis on prognosis. Such studies, however, require very large series of patients for adequate statistical evaluation and therefore generally depend on multicentre cooperation. Since more aggressive behaviour of tumour cells is generally associated with loss of differentiation antigens, it is of interest to search for and include antigens, which appear to be expressed in the more immature tumour types only in a multivariant analysis. Our data suggest that GICA may be an antigen of such nature.

The phenomenon of intercellular heterogeneity in tumours itself, however, is of interest to investigate in relation to more fundamental aspects of development and progression of neoplasia. In this context studies combining cell kinetics (as determined by autoradiography with ^3H -thymidine incorporation or by flow cytometry) with antigen expression might be rewarding to acquire information on the nature and significance of behaviour of neoplastically transformed tissue. This becomes even more pertinent in the light of the observation, that during their evolution the antigen expression of tumours may be liable to alteration (Baylin et al., 1978).

7. 4. Future perspectives of antigen expression studies in cell-origin-related classification of colorectal carcinoma

On the basis of the concept of maturation of neoplastically transformed cells in a particular stage of differentiation followed by clonal expansion of the arrested cell type, lymphologists nowadays have set out to classify malignant lymphomas according to the normal lymphoid cells to which they are related (Lukes et al., 1978; Stein, 1978). This approach has led to the distinction of neoplastic varieties within a group of morphologically indistinguishable tumours with prognostic and therapeutic implications.

Research in this field has been stimulated by the availability of a scale of in vitro functional tests for lymphocytes of different lineages and of distinct stages of maturation developed by immunologists. This set of tests could serve as a backbone for the comparison of antigen expression in normal lymphocytes and their purported derivative neoplasms. The incorporation of the functional check in this approach has led to the recognition, that many questions regarding the origin and function of neoplastic cells can be answered by comparison of their antigen expression profile with normal cells (for review see Mann et al., 1979). In this context the exquisitely rapid delineation of monoclonal antibody defined differentiation antigens in lymphocytes of the T and B lineage may be indicative (Kung et al., 1979; Reinhertz et al., 1979; Brooks et al., 1980).

If the results of these investigations in the field of malignant lymphoma research may be transferred to other neoplasms, this would imply that in regard of the typing and classification of colorectal cancer study of antigen expression possibly encompasses the most promising perspectives. Certain conditions, however, have to be considered in studies of this type in colorectal cancer.

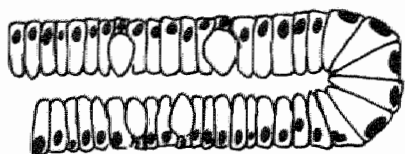
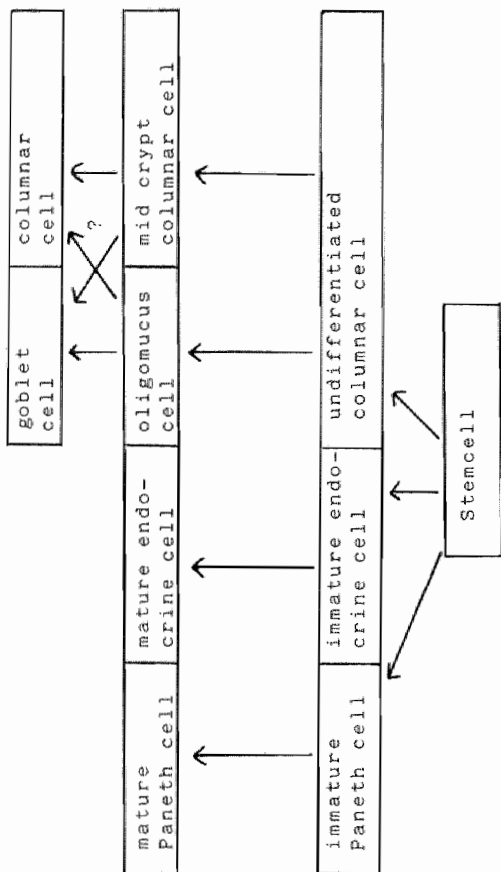
Firstly, a cell-origin-related approach, ideally, should be based upon a model of the derivation and differentiation of the main cell types of colorectal mucosa, but in this area of research certain controversies exist. At present the weight of balance of experimental data favours the

unitarian concept of origin of all intestinal epithelial cell types (Andrew et al., 1982). Kinetic experiments have delineated their interrelations and stages of development and maturation in the mouse colorectal mucosa, as represented schematically in fig. 1 (Chang and Leblond, 1971 a and b). With the uncertainty, whether these data are applicable to the human situation, this scheme could be used as at present the most acceptable concept of the interrelation in development and maturation of colorectal epithelium.

Secondly, there are ample indications from experimental and clinical data, that colorectal tumours do not allow a distinct classification according to one single type of differentiation, since during their evolution several lines of differentiation may be expressed in the clones of one tumour or even in individual tumour cells. Cox and Pierce (1982) elegantly showed in an experimental rat colonic adenocarcinoma of mixed composition, that the original display of differentiatinal characteristics reappeared during the development of a transplant tumour obtained after single tumour cell transplantation. "Stem cell" tumours displaying a variety of morphological and functional features have been described (Petrelli et al., 1981; Damjanov et al., 1983). Also mixed tumours composed of adenomatous and carcinoid elements are well known varieties featuring amongst colorectal carcinoids (Hernandez and Reid, 1969; Hernandez and Fernandez, 1976). As already mentioned colloid carcinomas frequently express lysozyme and neuroendocrine granules in addition to abundant mucus production (Arends and Bosman, 1983). Finally there are indications that in commonly occurring colorectal tumours (adenocarcinomas), SC immunoreactivity and mucus production frequently coincide (Arends et al., unpublished observations, see picture on the cover of this thesis).

Thirdly, a limitation is formed by the circumstance that in respect of colonic epithelial cells no in vitro functional tests have been developed to ascertain their stage of maturation and functional abilities. Therefore, in respect of colorectal carcinomas a cell-origin-related classification only relies on the comparison of the antigen expression profile of normal and neoplastic epithelium.

One way to attain the goal of obtaining antigens reflecting distinct stages of maturation and function would be to generate monoclonal antibodies by means of immunization of recipient animals with colonic epithelial cells of distinct stages of maturation. Since goblet cells and columnar cells mature and differentiate during the course of migration from the crypt base to the mucosal surface, selection of cells originating from different heights along the cryptal surface as immunizing agents would seem to be a rational approach to obtain antigens, which might reflect a particular stage of maturation. Techniques to acquire such a selection momentarily are available (Roediger and Truelove, 1979). It is conceivable, that on the basis of this approach "developmental" antigens can be detected on colonic epithe-



lial cells and their derivative neoplasms, as e.g. has been the case with T lymphocytes in the acquisition of monoclonal antibodies of the OKT series.

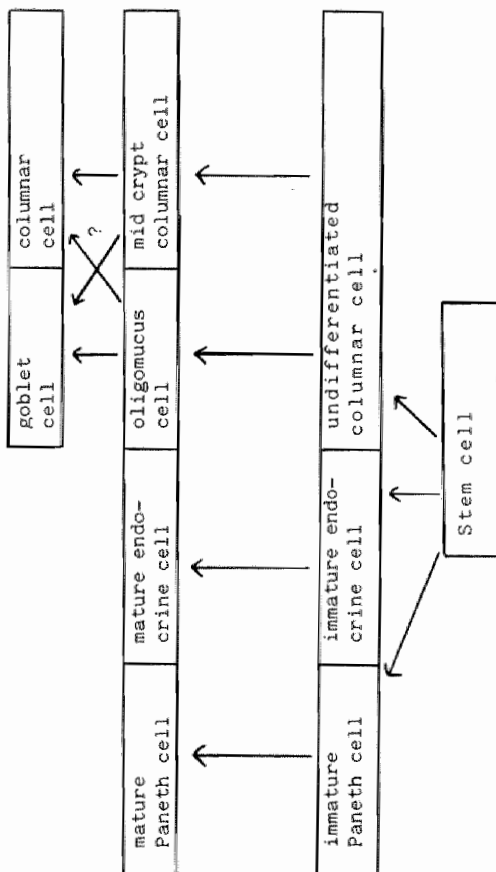
A systematic mapping of the expression of the secretory products and "developmental" antigens of the main colonic epithelial cell types in colorectal carcinoma could, therefore, given the above limitations, provide a spectrum with varying combinations or single expression of these antigens, from which in the context of the concepts outlined above the extent of pluripotency of the various colonic epithelial cell types in their respective stages of development and maturation could be read and according to which colorectal carcinomas could be positioned in a cell-origin-related scheme, as tentatively drawn in fig. 2.

7.5. Concluding remark - value of "developmental" antigens.

In this thesis the diagnostic and prognostic value of antigen expression in colorectal carcinomas has been evaluated against the background of the present limitations formed by the lack of antigen-specificity and the phenotypic heterogeneity of antigen expression.

From the literature and experimental data presented the perspectives of the study of antigen expression in colorectal carcinomas may seem rather futile. To this effect, however, one major point has to be made. Our study concentrated on "differentiation" antigens, but it should be realized, that the inclusion of "developmental" antigens may be crucial in the evaluation of the experimental and clinical value of tumour antigens in view of the notion, that many tumours, and most notably the poorly and undifferentiated variants, which generally behave most aggressively in patients and therefore pose a great challenge to medical scientists, do not express features and associated antigens of differentiation. Thus by studying "differentiation" antigens exclusively many tumours will remain refractory to efforts to characterize their behaviour in biological terms and also will be resistant to diagnostic and therapeutic procedures through detection and manipulation of antigenic display.

Therefore it should be apparent that the search for "developmental" antigens would greatly enhance the perspectives of the study of antigen expression in colorectal carcinomas.



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|---|
| pure mucus producing or exclusively SC positive carcinomas |
| adenocarcinomas with mixed mucus and SC expression |
| carcinomas displaying multiple morphological and functional features ? colloid carcinomas ? adenocarcinomas " Stemcell " tumours |
| undifferentiated carcinomas |

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SAMENVATTING

Dit proefschrift behandelt, aan de hand van een reeks immunocytochemische onderzoeken, enkele mogelijkheden en beperkingen van het onderzoek naar de expressie van macromoleculen (antigenen) in weefsel. Het onderzoek werd verricht op weefsel van patienten met colorectaal carcinoom.

Hoofdstuk I geeft een overzicht van de relevante literatuurgegevens betreffende de expressie van antigenen in colorectaal carcinoomweefsel. Het schetst het algemene kader, waarin de immunocytochemische onderzoeken hebben plaatsgevonden. De conclusie uit dit literatuuroverzicht is, dat het bepalen van antigeen expressie patronen in tumorweefsel van waarde kan zijn bij het karakteriseren en classificeren van primaire tumoren en hun metastasen (diagnose). Tevens mag op grond hiervan worden verwacht, dat de aan- of afwezigheid van bepaalde antigenen in tumoren van voorspellende waarde kan zijn voor hun gedrag bij patienten (prognose). Deze potentiële diagnostische en prognostische waarde wordt echter ernstig beperkt door twee factoren: antigenen, specifiek voor normaal of neoplastisch colorectaal weefsel ontbreken en bovendien vertonen tumoren in hun antigene samenstelling een aanzienlijke heterogeniteit.

Hoofdstuk II vermeldt de resultaten van een onderzoek naar de specificiteit van het "Gastro-Intestinal-Cancer-Associated antigeen (GICA)". Volgens voornamelijk op serologisch onderzoek en studie van cellijnen gebaseerde gegevens zou dit antigeen een distributiepatroon vertonen, dat beperkt is tot carcinomen en foetaal weefsel van de tractus digestivus. Ons onderzoek naar het voorkomen van GICA met behulp van een immunoperoxidase techniek bracht echter aan het licht dat dit antigeen op vele epitheel- en carcinoomsoorten, buiten het maag-darmstelsel, voorkomt. Met dit onderzoek wordt onderstreept dat het verrichten van immunocytochemische analyse op vers en gefixeerd materiaal van belang is in het kader van specificiteitsonderzoek van antigenen.

Hoofdstuk III behandelt een onderzoek naar correlaties tussen het expressiepatroon van GICA in colorectale carcinomen en enkele prognostisch belangrijke clinicopathologische variabelen, zoals de uitgebreidheid van de tumor en de histologische differentiatiegraad. Wij konden geen associatie tussen het expressiepatroon van GICA en de geteste variabelen aantonen. Daarom mag worden verwacht dat het expressiepatroon van dit antigeen in colorectale carcinomen geen aanwijzing geeft omtrent het gedrag van deze tumoren bij patienten.

Hoofdstuk IV beschrijft een onderzoek dat deze verwachting nader bevestigt. Uit dit onderzoek bleek dat geen signifi-

cante correlatie aanwijsbaar is tussen het GICA expressiepatroon en de overlevingsduur van patienten met colorectaal carcinoom. De gegevens suggereren echter dat GICA positieve cellen, een agressiever gedrag vertonen dan GICA negatieve cellen. Dit gegeven is uit biologisch oogpunt interessant. De relevantie ervan voor de klinisch pathologische praktijk is echter beperkt vanwege de heterogeniteit, die ten aanzien van de GICA expressie in een groot deel van de colorectale carcinomen gevonden wordt.

Hoofdstuk V vermeldt een onderzoek waaruit nog duidelijker blijkt dat de fenotypische heterogeniteit van antigeen expressie de prognostische waarde van het aantonen van weefselantigenen aanzienlijk beperkt. In dit onderzoek werden significante verbanden aangetoond tussen het expressiepatroon van secretair component (SC) in colorectale tumoren enerzijds en de uitgebreidheid en de histologische differentiatiegraad van de tumor en de overlevingsduur van patienten anderzijds. Tumoren met uniforme SC positiviteit werden in significant hogere mate aangetroffen in combinatie met een beperkte uitbreiding en een goede differentiatiegraad. SC positieve tumoren waren bovendien geassocieerd met een significant langere overleving. Tumoren zonder SC expressie daarentegen werden meer gevonden in de groep van carcinomen met uitbreiding door de gehele darmwand en een slechte differentiatiegraad. Deze tumoren gingen bovendien gepaard met een kortere levensduur van patienten. Bij carcinomen met een focaal, heterogeen SC expressiepatroon kon echter geen enkele relatie met stadium van uitbreiding van de tumor en histologische differentiatiegraad worden aangetoond. Het significante verschil in overleving tussen patienten met SC positieve en die met SC negatieve tumoren verdween bovendien, indien de focaal positieve tumoren bij de analyse betrokken werden. Uit deze bevindingen mag geconcludeerd worden, dat de SC expressie status in colorectale tumoren van prognostische betekenis is. Omdat zoveel tumoren een heterogene SC expressie vertonen, is de praktische betekenis ervan echter beperkt. De SC expressie status lijkt een bijdrage te kunnen leveren bij het stellen van de prognose van patienten met goed en matig gedifferentieerde colorectale carcinomen, doch blijft als enige prognostische parameter ondergeschikt aan het stadium van uitbreiding van de tumor.

Hoofdstuk VI stelt het fenomeen van de antigene heterogeniteit zelf aan de orde. Met behulp van een reeks antilichamen, die reageren met colorectaal carcinoomweefsel werd de mate van antigene heterogeniteit in primaire colorectale carcinomen bepaald. Tevens werd onderzocht of er verschillen in het antigeen expressieprofiel tussen primaire tumoren en metastasen bestaan. Lymfogene metastasen bleken even divers en heterogeen van antigene samenstelling als de primaire tumoren. Wij vonden echter aanwijzingen dat bij hematogene metastasen een verlies van antigenen kan optreden. De impli-

caties van deze bevindingen in verband met immunotargeting procedures worden eveneens in dit hoofdstuk besproken.

Hoofdstuk VII (epiloog) geeft een beschrijving van de perspectieven en beperkingen van het onderzoek naar de antigeen expressie in weefsel in het kader van onze onderzoeksresultaten. Daarnaast worden wegen aangegeven om de beperkingen te verminderen, die ontstaan door de twee voornoemde factoren: het ontbreken van antigenen specifiek voor colorectaal (carcinoom) weefsel en de heterogeniteit van de antigeen expressie.

Tenslotte wordt aandacht besteed aan de waarde, die het bepalen van weefselantigenen zou kunnen hebben voor een aan celorigine gerelateerde classificatie van colorectale carcinomen.

SUMMARY

In this thesis some perspectives and limitations of the study on antigen expression in tissue are considered in the context of a series of immunohistochemical investigations on colorectal carcinoma tissue.

Chapter I provides a review of the relevant data from the literature on antigen expression in colorectal cancer tissue and sketches the design of our experiments. From the data in the literature it can be concluded, that detection of antigen expression at the tissue level may be of value in the characterization and classification of primary tumours and their metastases (diagnosis). In addition it may be expected, that the presence or absence of certain antigens in tumours can be indicative for their behaviour in patients (prognosis). The lack of antigens specific for colorectal (cancer) tissue, as well as the phenomenon of antigenic heterogeneity in most tumours, however, pose severe limitations to this potential diagnostic and prognostic value.

Chapter II presents the results of a study on the specificity of Gastro-Intestinal-Cancer-Associated antigen (GICA). According to the data in the literature, mainly based on serological studies and exploration of cell-lines in culture, expression of this antigen was claimed to be restricted to neoplastic and fetal tissue of digestive tract origin. Our immunocytochemical exploration of GICA expression on a variety of tissue specimens, however, provided evidence that the antigen occurs in glandular epithelia and carcinomas of other than digestive tract derivation. The study therefore, underscores the importance of extensive immunocytochemical analysis on fresh and fixed tissues in the exploration of antigen-specificity.

Chapter III deals with a study to correlate the GICA expression status in colorectal carcinomas with clinicopathological variables of prognostic importance, such as the stage of tumour extension and the histological grade. Since the GICA expression status did not appear to be associated with these variables, it should be expected, that the prognostic value of determination of GICA expression is very limited.

This expectation is further confirmed by the study of Chapter IV, in which no significant correlation between the GICA expression pattern and the survival of colorectal cancer patients could be established. The suggestion from our data, that GICA expressing cells might show a more aggressive behaviour than GICA negative cells, is of interest from the biological point of view. The relevance of this phenomenon in clinicopathological practice, however, appears to be limited due to the occurrence of a substantial propor-

tion of colorectal carcinomas with a heterogeneous GICA expression pattern.

That the phenotypic of heterogeneity in antigen expression considerably interferes with the potential prognostic value of tissue antigen expression, is even more explicitly stressed in the study described in Chapter V. In this study significant associations could be demonstrated between the type of expression of secretory component (SC) in colorectal carcinomas on the one hand and the stage of tumour extension, the histological grade and the survival time in patients at the other hand. Uniformly SC positive tumours were significantly more often observed in the group of low-stage and low-grade carcinomas and in addition showed significantly longer survival periods in patients. In contrast, tumours without SC expression were associated with high-stage and high-grade carcinomas and demonstrated shorter survival periods in patients. Tumours with a focal, heterogeneous SC expression pattern, however, failed to show any correlation with stage and with grade, whereas their inclusion in the analysis of survival data obscured the significant difference in patient survival between SC positive and SC negative tumours. Although from these observations it can be concluded that the SC expression status is of prognostic significance in colorectal carcinomas, the occurrence of a substantial proportion of tumours with a heterogeneous SC expression pattern attenuates its practical value. SC expression as such appears to be a valuable prognostic indicator in patients with well- or moderately differentiated colorectal carcinomas, but as independent prognostic parameter remains inferior to that of the stage of tumour extension.

In Chapter VI the phenomenon of antigenic heterogeneity is highlighted. Through the application of a series of colorectal cancer-reactive antibodies the extent of antigenic heterogeneity in primary colorectal carcinomas was determined. At the same time the question, whether metastases due to the clonal selection purportedly occurring in metastasis might demonstrate a more restricted or different antigen expression profile, was investigated. Lymphnode metastases and primary tumours appeared to be equally diverse and heterogeneous of antigenic composition, whereas hematogenous metastases might show a tendency to loose expression of some antigens. The implications of heterogeneity in antigen expression and possible loss of antigens in regard to immunotargeting procedures are also discussed in this chapter.

In Chapter VII (the epilogue) the perspectives and limitations of the study of tissue antigen expression are reviewed in the context of the results obtained in our investigations. Ways which might alleviate the impediments formed by the (current?) lack of colorectal (cancer)tissue-specific antigens and the occurrence of antigenic heterogeneity are indicated. Finally the potential value of tissue antigen detection for a cell-origin-related classification of colorectal carcinomas is considered.

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